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Daniel J. MacPhee *Editor*

The Role of Heat Shock Proteins in Reproductive System Development and Function

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222

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Preface

Heat shock proteins (HSPs) are a large family of very conserved proteins with important roles in cellular homeostasis and cytoprotection from chronic or acute stressors. As molecular chaperones they can participate in physiological processes and protect proteins from aggregation, promote their proper folding, and help mediate protein degradation under physiological or stressful conditions. The HSPs are grouped under HUGO Gene Nomenclature Committee guidelines (former names in parentheses) as HSPA (HSP70), HSPB (small HSP), HSPC (HSP90), HSPH (HSP110), DNAJ (HSP40), and the human chaperonin families (HSP60 and CCT). Recent research has uncovered new and exciting roles for these molecular chaperones in reproductive system development and function. Due to the paucity of information on this subject, prominent researchers have provided reviews in this issue highlighting the most current findings on HSP expression and function in the reproductive system.

HSPs are proteins with many roles or talents in organs and cells; hence, I like to refer to them as *renaissance proteins*. However, this does present a significant challenge to understanding their specific roles in the regulation of developmental events. The breadth of the work being conducted on these proteins within the field of Reproductive Sciences is exemplified by the contributions within this series. ***Dr. Elisabeth Christians*** reviewed HSPs and their involvement in the maternal contribution to oogenesis and early embryogenesis. She particularly reviewed the literature on this topic in key organisms such as *Drosophila melanogaster*, *Caenorhabditis elegans*, and vertebrates including *Mus musculus*. Heat shock factors (HSF) are key transcription factors that regulate HSP expression, and ***Drs. Wieslawa Widlak and Natalia Vydra*** reviewed the roles of these factors in mammalian spermatogenesis, particularly HSF1 as a proposed quality control factor in male germ cells. Remaining on the topic of spermatogenesis, ***Dr. Brett Nixon and colleagues*** have provided an examination of the structural and biochemical characteristics of HSPA2 and how it allows this HSP to direct the morphological differentiation of male germ cells during spermatogenesis and their functional transformation during sperm maturation.

The HSPB family comprises eleven members with many induced by physiological stressors. In addition to being chaperones, these proteins also play important roles in cytoskeletal rearrangements and immune system activation. These same processes are important for the uterine smooth muscle or myometrium during pregnancy as it changes from a quiescent to a powerful and contractile tissue at labor. ***Drs. MacPhee*** and ***Miskiewicz*** summarized current knowledge on the expression of HSPB family members in the myometrium during pregnancy and the possible roles of these proteins during myometrial programming and transformation of the myometrium into an immune regulatory tissue. Continuing with a focus on pregnancy and parturition, ***Dr. Steven Witkin and colleagues*** reviewed the relationship between HSPA1A (inducible HSP70) and autophagy during gametogenesis, pregnancy, and parturition and how changes in that relationship can contribute to infertility and complications of pregnancy such as preeclampsia and preterm birth.

Reproductive immunology is another field exhibiting ever-growing interest in HSPs.

Dr. Eusebio Pires examined the evidence of a role for HSP90 in autoimmune ovarian failure, where autoantibodies to it have been observed in patients, which has been correlated to infertility. Finally, the HSP70 family member glucose-regulated protein or GRP78 (also known as HSPA5) is one of the best characterized endoplasmic reticulum chaperone proteins. ***Dr. Cheng Zhang*** summarized the biological or pathological roles and signaling mechanisms of GRP78 during female reproductive processes. In total, the scientific knowledge reviewed in this issue will promote additional cutting-edge basic and translational research on heat shock proteins in reproductive sciences. This could lead to advancements in the treatment of many instances of reproductive system dysfunction.

Saskatoon, Saskatchewan, Canada Daniel J. MacPhee

Abstract

Heat shock proteins are a large family of very conserved proteins that have important roles in the maintenance of cellular homeostasis and cytoprotection from chronic or acute stressors. These proteins are molecular chaperones, meaning they can participate in physiological processes and protect proteins from aggregation, promote their proper folding, and help mediate protein degradation under physiological or stressful conditions. They also rely on an ever-growing number of identified co-chaperones to modulate their function. The heat shock proteins are grouped under HUGO Gene Nomenclature Committee guidelines (former names in parentheses): HSPA (HSP70), HSPH (HSP110), HSPC (HSP90), DNAJ (HSP40), HSPB (small HSP), and the human chaperonin families (HSP60 and CCT). Importantly, recent research is uncovering new and important roles for these molecular chaperones and co-chaperones in reproductive system development and function. Due to the paucity of reviews on this subject, this proposed series aims to be timely and promotes additional basic and translational research on these proteins in reproductive system development and function within the fields of Anatomy, Embryology, and Cell Biology. The breadth of the work being conducted within Reproduction is exemplified by the contributors to this series who will provide reviews on Grp78 roles in female reproduction (Dr. C. Zhang), small heat shock proteins/co-chaperones as players in uterine smooth muscle function (Dr. D.J. MacPhee), the role of heat shock proteins in sperm function (Dr. B. Nixon) and maternal contribution to oogenesis and early embryogenesis (Dr. E. Christians), heat shock factors and testes development (Dr. W. Widlak), HSP90 in ovarian biology and pathology (Dr. E. Pires), and the role of HSP70 in regulation of autophagy in pregnancy and parturition (Dr. S. Witkin).

Contents

Heat Shock Proteins and Maternal Contribution to Oogenesis and Early Embryogenesis

Elisabeth S. Christians

Abstract Early embryos develop from fertilized eggs using materials that are stored during oocyte growth and which can be defined as maternal contribution (molecules, factors, or determinants). Several heat shock proteins (HSPs) and the heat shock transcriptional factor (HSF) are part of the maternal contribution that is critical for successful embryogenesis and reproduction. A maternal role for heat shock-related genes was mainly demonstrated in genetic experimental organisms (e.g., fly, nematode, mouse). Nowadays, an increasing number of "omics" data are produced from a large panel of organisms implementing a catalog of maternal and/or embryonic HSPs and HSFs. However, for most of them, it remains to better understand their potential roles in this context. Existing and future genome-wide screens mainly set up to create loss-of-function are likely to improve this situation. This chapter will discuss available data from various experimental organisms following the developmental steps from egg production to early embryogenesis.

1 Introduction

Heat shock proteins (HSPs) were originally defined as encoded by genes transcriptionally activated following a thermal stress (Lindquist and Craig 1988). While HSPs were originally grouped in families according to their molecular weight and some common molecular features, their nomenclature can be confusing. To find accurate additional information about human & mouse *HSP* genes, which are used as references, the reader can look at the nomenclature information collected by Kampinga and colleagues (2009) in addition to the organism databases and websites [Flybase (Attrill et al. 2016); Wormbase (Howe et al. 2016); ZFIN (Howe et al. 2013); Xenbase (Karpinka et al. 2015); Mouse genome informatics/MGI (Eppig et al. 2015)]. Rules for species-specific gene and protein

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nomenclatures, which can be found in the organism-related websites are followed as accurately as possible in this chapter.

Numerous *HSP* genes (but not all of them) are controlled by transcriptional factors named heat shock factors (HSFs). Along the evolutionary tree, multicellular organism genomes contain either one single *HSF* or multiple paralogs, which exert redundant and/or specific functions (Akerfelt et al. 2010). This chapter is centered on HSPs and HSFs and their roles during animal oogenesis (i.e., oocyte formation) that produces fertilizable eggs competent for successful development and is linked to early embryonic development. Despite their importance, non-*HSP* targets of HSFs will not be discussed in this chapter.

Most HSPs are chaperones meaning that they interact temporarily with other molecules or proteins to influence their fates (folding, trafficking, degradation). HSPs are frequently presented as coded by housekeeping genes and because they exist as families, several members of the same family can exert redundant functions. These features often lead to a lack of consideration for HSPs as important proteins whose loss-of-function can have dramatic consequences for living organisms. In contrast with this viewpoint, this chapter will present several examples demonstrating that HSPs with their regulators, HSFs, are important for reproduction, a critical function for species maintenance.

Nowadays, messenger RNA and protein expression can be assessed in nearly all cell types or developmental stages (with the remaining limitation of sensitivity of detection) so that a lot of information about HSP and HSF expression can be obtained from existing databases. For this chapter, we searched several of those databases and compiled the available results. Of course, expression profiles are mainly descriptive data unless they are compared between stages or developmental conditions in which cases they can suggest some mechanisms of regulation. Finally, to determine the functional importance of HSPs and HSFs in eggs and early embryos, we looked for loss- and gain-of-function experiments, which are available in a relatively limited number of experimental organisms.

In this chapter, we focus on two invertebrates (*D. melanogaster*, *C. elegans*) and three vertebrates (*X. laevis*, *D. rerio*, and *M. musculus*) as most of the literature published on heat shock (HS)-related genes during oogenesis and early embryogenesis exploited those organisms.

2 Invertebrates

2.1 Drosophila melanogaster

Drosophila melanogaster is the historical experimental organism, which was instrumental in defining the classical heat shock response that subsequently led to the discovery of genes encoding HSPs and HSFs [reviewed in Ritossa (1996) and in Lindquist and Craig (1988)]. Despite numerous studies investigating HSP and HSF

roles and the wealth of information on gene function in *D. melanogaster*, their specific roles during the fly oogenesis remain to be further clarified.

In *D. melanogaster*, oogenesis is divided into 14 stages based on morphological criteria and the organization of follicle, nurse cells and oocyte [described in Bastock and St Johnston (2008), McLaughlin and Bratu (2015)] (Fig. 1a). Oocytes emerge during sequential divisions of one germinal stem cell leading to the production of 15 nurse cells and one single differentiating oocyte. Then the oocytes grow accumulating maternal factors and determinants mainly from their sisters, the nurse cells, which undergo endoreplication and exhibit larger nuclei. Oocyte meiotic maturation occurs between stage 12 and 13 after nurse cells have transferred their entire cytoplasm into the oocyte. Metaphase1 (MI) oocytes are activated as they progress through the uterus and they are fertilized before being laid. Within 2.5 h after fertilization and 14 cell cycles, embryos (cellular blastoderm) become transcriptionally active (Fig. 1a).

Heat shock-related genes (*hsps*, *hsf*) are expressed during oogenesis and early development, but they do not seem to be regulated along the major steps of oocyte preparation such as maturation or activation. As described in the following paragraphs, control of their expression and function is rather cell and gene specific.

Oogenesis and larval development were severely affected by Hsf loss-of-function experiments (Jedlicka et al. 1997). Four different mutations were generated and three of them impacted oogenesis (Fig. 2a). Germ cell division and differentiation were not affected by *hsf* mutations, but the second part of oogenesis (growth phase, vitellogenic phase) was significantly impaired leading to female infertility. It was suspected that endoreplication in nurse cells did not occur normally causing subsequently the abnormal development of the oocyte. *Hsp83* expression was investigated in *hsf* mutants, using a *lacZ* reporter gene but the signal was not visibly affected. It is worth mentioning that this approach does not really allow quantitative evaluation. Nevertheless, it was concluded that Hsf critical function during oogenesis was linked to non-*hsp* target genes. *Hsf 1–3* fly mutants demonstrated that Hsf was required for oogenesis, but in *hsf4* thermosensible mutant oogenesis could be completed so that the heat shock-related function of Hsf appeared to be dispensable for egg production. Developmental role and heat shock ability were overlapping only during the 1–2nd instar larval stage. Therefore, because of the distinct effect of the *hsf* mutations on oogenesis and heat shock response, Jedlicka and colleagues proposed that «*hsf*-dependent phenotypes observed during the 1–2nd instar larva and the oogenesis are genetically separable and appear not to be mediated through the induction of *hsps*, implicating a novel action of Hsf that may be unrelated to its characteristic function as a stress-responsive transcriptional activator» (Jedlicka et al. 1997).

Beside the fact that *hsp83* could not be confirmed as an important Hsf target gene (Jedlicka et al. 1997), there is little information about how this gene is regulated under normal developmental conditions. At the posttranscriptional level, it was shown that the localization of *hsp83* transcripts was under a selective developmental control. *Hsp83* transcripts undergo a degradation/protection process to be maintained at the posterior pole following egg activation and fertilization, two

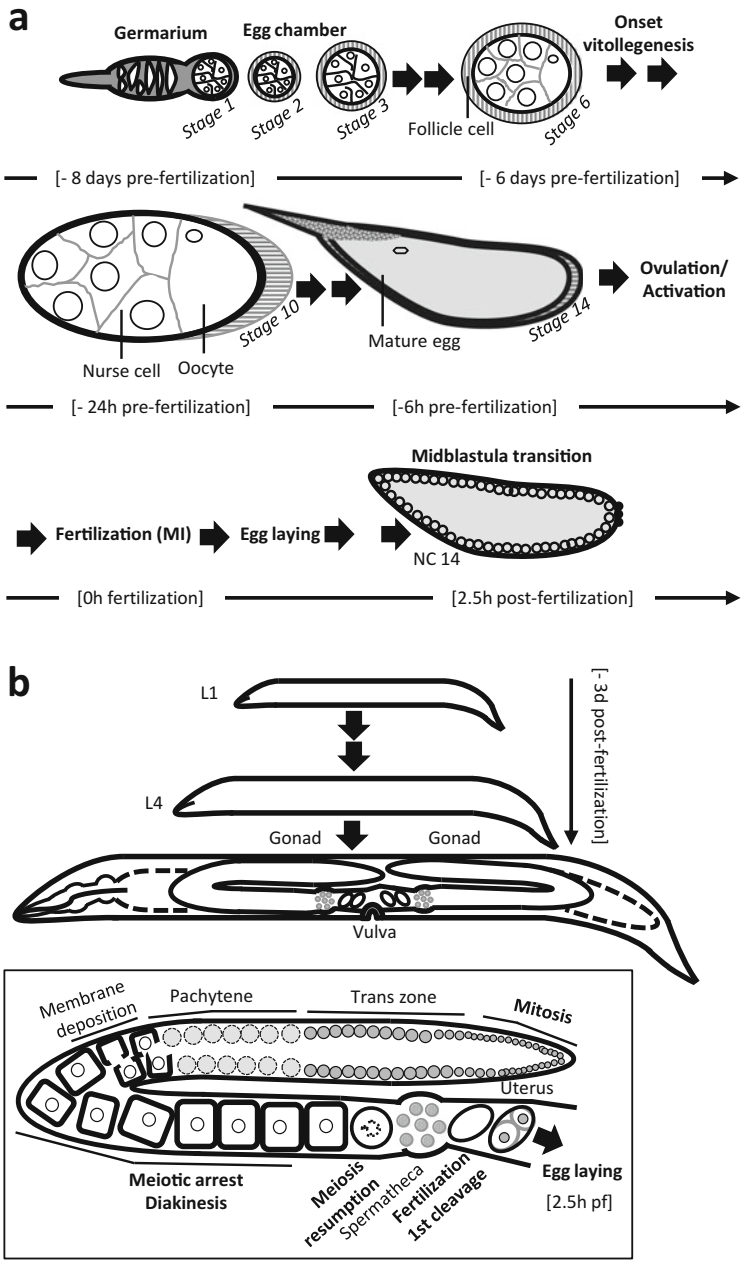

Fig. 1 Schematic illustration of oogenesis and early development in invertebrate experimental organisms (*Drosophila melanogaster*, *Caenorhabditis elegans*). (**a**) *D. melanogaster* oogenesis is localized in the ovariole, and the ovary is made of several ovarioles (not shown). One ovariole is shown with the succession of compartments from the germarium containing germline stem cells to the egg chamber chain corresponding to successive stages up to the mature egg. Major stages are depicted (stage 6: vitellogenesis; stage 10: towards meiosis resumption; stage 14: mature egg arrested at metaphase I). After fertilization, a series of rapid divisions followed by cellularization

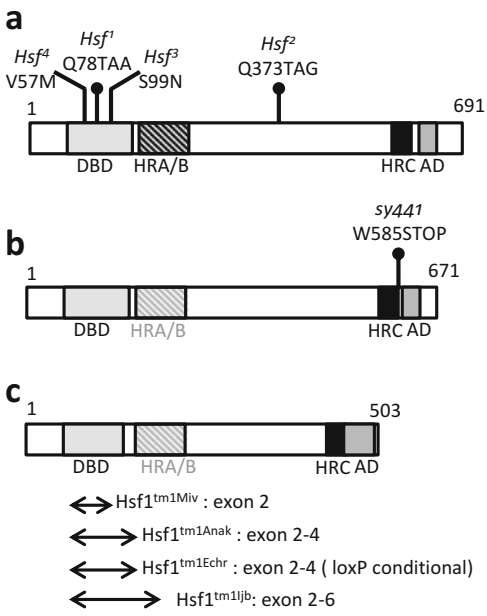

Fig. 2 Heat shock factor (*HSF* or *HSF1*) mutations. (**a**) Four mutations were isolated in *D. melanogaster*. *Hsf4* was not directly involved in reproductive phenotype. Line with bullet head indicates stop codon. (**b**) *C. elegans Hsf* mutant strain is named *Sy441* and corresponds to a stop codon shortening the protein by 86 residues. (**c**) *M. musculus Hsf1* knockout lines were created by deletion of various portions of the gene as indicated by *horizontal arrows*. See text for additional information and references

developmental steps, which trigger regulated degradation of maternal transcripts (Semotok et al. 2005). Zygotic transcripts accumulate in the anterior pole. Despite some insights into the molecular mechanisms allowing such regulated degradation, the functional meaning of preserving *hsp83* mRNA at these locations remains to be better understood (Semotok et al. 2005). A series of *hsp83* mutant flies demonstrated the essential role played by the encoded Hsp90 protein and its requirement for viability (Yue et al. 1999). Some trans-heterozygotes exhibited a very low viability (<1%) but when they survived, females were either weakly fertile or sterile. The mutant combining R48C and S592F mutations exploited by Pisa and colleagues was sterile due to severely altered oogenesis, which could not progress beyond stage 9 (Pisa et al. 2009). Their study identified Hsp83 as an interactor of Cup involved in protein translation machinery during oogenesis, and they showed that *Hsp83* mutant heterozygosity increased the severity of oogenesis defects due to *cup* mutant context. Hsp83 was also found to be involved in the localization of specific maternal transcripts such as *nanos* (Song et al. 2007). Since *Drosophila* development is characterized by the precise localization of maternal transcripts in

Fig. 1 (continued) leads to the formation of a blastoderm. At this stage, the midblastula transition occurs. (**b**). *C. elegans* oogenesis is localized in gonads whose formation is initiated during larval stages. Adult worm is presented with the anatomical organization of the hermaphrodite gonads. Schematic illustration (boxed) shows the gonad at higher magnification with the successive steps of female gamete production (2.5 maturing egg/per gonad/h) up to fertilization, early development. See text for additional information and references. pf: post-fertilization

the egg in order to establish the future embryonic axes, embryos bearing distinct *hsp83* mutations exhibited disorganized antero-posterior axis and segments because *nanos* transcripts were mislocalized and the protein could not be properly accumulated.

Regarding other HS-related genes, Cobreros and colleagues using gain- and loss-of-function strategies revealed the role played by *dnaJ*/*hsp40* and *hsp70* genes in border cell (BC) migration (Cobreros et al. 2008). BC migration is dynamic and well guided allowing this subpopulation of follicular cells to delaminate from the anterior part of this epithelium to progress between nurse cells up to the anterior-dorsal pole of the oocyte from stage 9 to 10. This process has been often presented—beyond fly and developmental biology—as a model for epithelial-mesenchymal transition (EMT), a hallmark of metastatic cancer cells, reinforcing interest in a better understanding of its molecular actors. In fly, this process is important because it contributes to the organization of the egg chamber, which is critical to localize maternal factors. In their study, Cobreros and colleagues suggested that DnaJ/ Hsp40 and Hsp70 would be needed to support actin cytoskeleton reorganization under the control of PVR (PDGF/EGF-related receptor) (Cobreros et al. 2008). As the *Drosophila* genome includes six *hsp70*-related genes (see FlyBase, Attrill et al. 2016), which were all deleted in this study, it is difficult to conclude whether all of them are required or not and in this later case, which ones would be necessary. Two other studies based on genetic screens revealed that *hsp70–4* (CG4264) is an important contributor to production of a healthy egg. Jia and collaborators searched for genes involved in Notch-mediated follicle cell differentiation using an RNAi screen (Jia et al. 2015) and among 33 selected genes, they found *hsp70–4*, already previously described as a Notch trafficking modifier (Hing et al. 1999). So *hsp70–4* impacts the transition from mitosis to endocycle during mid-oogenesis through the Notch-Delta pathway linking follicle cells and germline. *Hsp70–4* had already been previously identified as a maternal effect gene during a screen performed by Perrimon and colleagues (1996). Deficiency in *hsp70–4* in oocytes induced a mutant phenotype in the offspring, which affected the abdominal segments and looked like pair-rule related. Thus, Hsp70–4 plays multiple roles depending on which cell in the egg chamber is mutant. A second gene in the Hsp70 family, *hsp70–5*, was found to be required for female fertility. This result was obtained during a screen for genes involved in regulating transposon silencing in the oocyte. The study by Czech and collaborators classified *hsp70–5* among the 100 strongest hits but did not provide more information about the role played by the chaperone (Czech et al. 2013). In contrast, transposon silencing during spermatogenesis requires another chaperone, Hsp83 (Specchia et al. 2010), which was not found in the oocyte screen performed by Czech and colleagues even if this protein is abundantly expressed in female gametes. This suggests that this function is molecularly supported in a different way during oogenesis and spermatogenesis.

As described above, the organization of the egg chamber requires complex and dynamic interactions between the follicle cells and germ cells, either nurse cells or oocyte. The Hsp60 family counts four genes and *hsp60C*, which is required to get

viable flies, is also the most expressed gene in the three types of ovarian cells. Therefore, even in presence of the three other wild-type *hsp60* genes, *hsp60C* mutation can significantly reduce the amount of Hsp60 protein and provoke an efficient loss-of-function condition. To avoid the *hsp60C*-associated lethality (10% survivor flies), mutant clones were created in egg chambers, which were severely affected starting from stage 8. Hsp60C-deficient function was shown to perturb the actin and microtubule-related cytoskeleton. This induced numerous alterations, in particular in the cohesion and organization of the follicle cells, which in turn impact, for example, the microtubule organizing center (MTOC) and nuclear position in oocytes (Sarkar and Lakhotia 2008). This was consistent with the Hsp60C accumulation in the cytoplasmic region where the oocyte is in contact with nurse cells, a domain where a new MTOC is formed.

Small heat shock proteins are encoded by seven genes grouped in close genomic proximity. Transcripts are present in ovarian cells: follicle, nurse cells, and oocytes. Nurse cells synthesize the mRNAs, which are transferred to the oocyte. The oocyte undergoes maturation and then activation, two important steps in the transition from oocyte to embryo (Fig. 1a). Based on proteomic analysis, Hsp27 is the only Hsp to be upregulated during maturation, the other small Hsps remain unchanged at the protein level during these transitions (Kronja et al. 2014). Oocyte transcripts can be maternally transmitted to the embryo where they are distinctly degraded or maintained (*hsp26* degraded by stage 1–3, *hsp27* maintained in pole/germline cells) (Morrow and Tanguay 2012). Female fertility is not severely affected by specific mutations in small *hsp* genes, and this can be explained by the potential redundancy between the different members of the family.

To comment on HSR—omega heat shock gene, which produces a noncoding RNA, its roles remain to be better understood even if it is a well-known genomic site induced by heat stress in *D. melanogaster* (Pardue et al. 1990). It is ubiquitously expressed except in the egg chamber where only the nurse cells produce the transcript. It seems to be specifically degraded so there is no maternal transmission and it requires the zygotic transcription to be detected in embryos (Bendena et al. 1991). Its role in nurse cells could be related to the protein synthesis activity (Johnson et al. 2011) and would remain secondary since existing mutants do not exhibit any sterility.

Expression profile and mutant analyses reported so far were performed in normal conditions, meaning in absence of defined stress. Few studies investigated the heat shock response in ovarian cells in *Drosophila*. Based on quantification of in situ hybridization, it was shown that follicle cells exhibited a strong induction of various *hsp* mRNAs (*hsp70*, *hsp83*, and small *hsps*). Following HS, there was a significant increase of *hsp70* transcripts and much less of *hsp83* mRNA as it was already present at a higher level (Ambrosio and Schedl 1984). Wang and Lindquist followed the response of the nurse cells, which is maintained up to stage 10 (Wang and Lindquist 1998). Then they progressively become incompetent as Hsf is relocated in the cytoplasm. Treatment by heat shock is not able to release oocyte transcription from its quiescent status even if Hsf is found located in the oocyte nucleus, potentially able to bind the specific site in the promoter of *hsps* named a heat shock element (HSE), two features characteristic of the HSR. It seems

that *Drosophila* oocyte is unable to increase its content in Hsp/chaperone following heat stress even if it is connected to competent nurse cells. After fertilization, embryos are transcriptionally silent during 2.5 h and thus unable to induce *hsp* gene transcription following stress. They acquire the ability to mount a classical heat shock response soon after the cellular blastoderm stage (NC14, Fig. 1a) (Eberlein and Mitchell 1987).

2.2 Caenorhabditis elegans

Caenorhabditis elegans belongs to the protostome group as does *Drosophila melanogaster*. It also shares the extensive use in laboratories as a genetic experimental model. Nevertheless, this organism is rather distant regarding oogenesis processes, early development, as well as many other biological features (e.g., two genders: male and hermaphrodite with successive spermatogenesis and oogenesis, neuro-endocrine control of stress response). When fully formed, gonads in *C. elegans* worm are organized as a U-turn tube producing oocytes connected to a spermatheca and then joined to a single uterine structure and vulva (Fig. 1b). During *C. elegans* development, oogenesis occurs following spermatogenesis in the same animal, which is therefore able to produce self-fertilized embryos as long as the defined number of spermatozoa ($n = 160$) is available. Following a five-day period, hermaphrodites still generate oocytes but they require cross-fertilization by a male. Female gamete production starts during late larval stage. Along the gonad tube, they first multiply by mitosis, then enter meiosis, and block at the end of prophase I. Egg maturation is induced as oocytes reach the spermatheca at the end of the gonad (distal extremity). Following fertilization, embryos perform several cleavages inside the uterus before being laid. This last step requires functional muscles and neurons while it responds to various environmental conditions (Altun 2009; Kimble and Crittenden 2007). Following the gamete and embryo production chain, egg laying itself is under a complex network of genes including heat shock-related genes as described below.

Although the accessibility to the gametes might be difficult in *C. elegans* due to the organization of the gonads, transcriptomes and proteomes specific to either oogenesis or spermatogenesis could be analyzed. This was possible by means of gonads producing only one type of gametes due to specific mutations. Of course one has to assume that the respective mutations did not significantly alter the profile of gene expression (Ortiz et al. 2014). Ortiz et al. (2014) found that numerous heat shock-related genes (hsf-1, 16 hsps, and 28 dnaj genes) were expressed at a detectable level in gonads. However, a limited number of genes were significantly enriched in oogenic versus spermatogenic gonads: hsf-1, hsp-12.6, and three hsp40-related genes: dnj-7, 14, 23 (Ortiz et al. 2014). At the proteomic level, we identified two studies reporting HS-related protein expression in oogenic gonads that were generated from different mutants (Chik et al. 2011; Tops et al. 2010). When compared to masculinized gonad, feminized gonads contained higher levels of

HSP-16.1, HSP-6, DAF-21, DNJ-12, and HSP16–48 (Tops et al. 2010). In their study, Chik and collaborators reported the presence of the following proteins: HSP-1 (HSP70), HSP16–1,-2,-41,-48, HSP-3 (GRP78), HSP-6 (HSP70), DAF-21 (HSP90), and DNJ-12. At a quantitative level, DAF-21 appeared to be the most abundant HSP followed by the two members of the HSP70 family. Those studies are relatively difficult to compare as they exploited different mutants and different technologies to generate their results. Nevertheless, they identified a distinct series of heat shock-related genes expressed in female or feminized gonads suggesting that oocytes need a defined set of chaperones. To validate the importance of these expression data on a functional basis, worm databases can be searched for related mutants or RNA-I knockdown.

In 2004, a screen that was designed to identify suppressors of a heat shock inducible transgene selected a mutation in *hsf-1* (*sy-441*) (Hajdu-Cronin et al. 2004) (Fig. 2b). The *hsf-1* mutation truncated the carboxyl terminus of HSF, eliminating the transactivation domain. Its phenotype was characterized by anomalies in larval development, egg-laying behavior, and longevity, in addition to deficiency in the heat-shock response. The authors indicated that "the egg-laying defect of *hsf-1 (sy441)* was suppressed in progeny of parents that were deprived of food" (Hajdu-Cronin et al. 2004). This suggests that environmental conditions further modulate the anomalies due to this mutation. RNAi-induced loss-of-function of HSF-1 was investigated by several investigators under various experimental conditions. This resulted in either larval arrest or globally unhealthy worms, which exhibited reproductive defects including the "bag of worms" phenotype (Walker et al. 2003). Although the expression data had shown that HSF-1 is a maternal factor in worms, the role played by HSF-1 in nematode oocytes themselves remained to be better understood.

The mutation *daf-21* (corresponding to *hsp90*, an HSF target gene and interactor) provokes the constitutive formation of the dauer larva in absence of environmental signals (Vowels and Thomas 1994). Further analysis of this strain revealed a reduced brood size, and this observation prompted new studies focusing on their reproductive ability. Inoue and collaborators first described the localization of daf-21 expression in gonads and then they undertook to phenocopy *daf-21* mutation by using RNA interference (Inoue et al. 2003, 2006). Acting on the adult worm, daf-21 RNAi disrupted the resumption of meiosis in oocytes, which became endomitotic. Because this phenotype was also reminiscent of the defaults induced by wee-1.3 RNAi, it was speculated that DAF-21 would be required to stabilize WEE-1.3 and thus contribute to maintain CDK1 inhibitory phosphorylation. When daf-21 was downregulated, WEE-1.3 would be degraded and CDK1 activated in an inappropriate way (Inoue et al. 2006).

RNA interference is a very powerful approach to induce loss-of-function in *C. elegans* and beyond the two previous examples, HS-related genes were targeted by numerous RNAi sequences exploited in several large screens (Green et al. 2011; Sonnichsen et al. 2005) and compiled in Wormbase (Howe et al. 2016). Among the 50 or so heat shock genes found in the *C. elegans* genome, about ten genes targeted

Table 1 RNAi corresponding to HS-related genes–Hsp exhibiting oogenesis and/or developmental phenotype (Wormbase)

Gene	Phenotypes (stages)			
Name ID WBGene	Germline/Germ cell	Gonad	Embryo	Larva
hsf-1 00002004	ND	Abnormal	Bag of worms	Arrest lethal
hsp-1 (Hsp70A) 00002005	Abnormal	Abnormal vesiculated	Dead egg laid	Arrest lethal
hsp-2 00002006	Abnormal	Maternal sterile	Dead egg laid	Lethal (early)
hsp-3 (BIP) 00002007	ND	ND	ND	ND
hsp-4 (BIP) 00002008	Oocyte (no nucleus)	Abnormal, vesiculated	Lethal	Lethal
hsp-6 DNAK/HSP70 00002010	Abnormal oocyte	Abnormal	Arrest reduced number	–
hsp-60 GroE 00002025	Abnormal	Abnormal, sterile	Dead egg laid	Arrest
hsp12.2 00002011	–	Maternal sterile	–	–
hsp16.41 00002018	–	–	–	Arrest
dnj-11 DNAJC2 00001029	Abnormal	–	Lethal	–
daf-21 HSP90 cyto. 00000915	Endomitotic oocyte	Abnormal	Lethal	Arrest lethal

by RNAi were responsible for reproductive phenotypes either at the level of the germ cell, gonad, or embryo/larva development (Table 1).

Regarding the heat shock response and proteostasis, *C. elegans* embryos could activate HS-related promoters as early as 12-cell stage (Zhu et al. 1998). This worm also revealed puzzling interactions between fecundity/reproduction/germ cells and age-related decline in HSR (Labbadia and Morimoto 2015). A few hours after the onset of reproductive maturity in young adults, expression of the JMJD3.1 demethylase decreases and subsequently methylation of H3K27 increases at several loci including HSF target genes. As a consequence, HSR efficiency is reduced. Although genetically established, the link between reproduction and the molecular events remains to be better understood. The number of germline stem cells (GSC) appeared to be critical since a smaller GSC population allowed a better maintenance of HSR. Nevertheless, this was not a cell-autonomous phenomenon because it required a signaling pathway between GSC and somatic cells to trigger the molecular modifications described above (Labbadia and Morimoto 2015).

3 Vertebrates

The vertebrate subphylum includes five main classes (fish, amphibian, reptiles, bird, and mammals), which also differ by their ability to maintain their stable temperature: birds and mammals are homeothermic while the three other classes are mainly poikilothermic. Eventually, this physiological characteristic should be kept in mind when studying the effect of temperature on cells from species belonging to these different groups and the profile of HS-related gene expression. Fish, amphibian, and mouse are the three most popular experimental vertebrate organisms and a previous review described in detail the expression pattern of heat shock genes during their embryonic development (Rupik et al. 2011). Complementing this description and as a part of a HSF dedicated minireview series, Abane and Mezger (2010) focused their paper on the HSF transcription factor family during gametogenesis (male and female) and embryonic development, mainly in mammals. Since then, major progress has been made in transcriptomic/proteomic analysis of gametes and embryonic cells while, in comparison, fewer studies investigated the functional aspects of heat shock (HS)-related genes.

3.1 Danio rerio

D. rerio or zebrafish is a tropical freshwater fish, which became popular in laboratories interested in developmental biology but also in reproduction (Hoo et al. 2016). Zebrafish females can produce fully developmentally competent eggs from about 3 months to 1.5 years of age (Fig. 3a). Reproduction and egg quality are under complex interactions between age, animal size, and other environmental conditions but on average, females can spawn up to 200 eggs two or three times a week. Furthermore, as a genetic model organism, zebrafish was exploited for large functional screens (Kettleborough et al. 2013) and, in particular, to identify maternal effect mutations (Abrams and Mullins 2009). Maternal product deposition is important for embryonic development but as far as we know, those screens have not yet revealed any involvement of HS-related genes even if they are highly expressed in mature, fertilizable eggs (e.g., Rauwerda et al. 2016).

Interesting information about expression of HS-related genes can be found in at least three transcriptomic databases generated either from mature eggs (Rauwerda et al. 2016) or early embryonic development (Aanes et al. 2011; Harvey et al. 2013) (Table 2). Those studies had different purposes and were performed according to different methodologies so they do not provide fully equivalent information regarding the same set of HS-related genes. Furthermore, there were some discrepancies between the data they reported. For example, *hspe1* transcripts could not be detected in mature eggs (Rauwerda et al. 2016) but were measured as abundant at the 2-cell stage (Harvey et al. 2013), and Hspe1 protein was listed in oocyte proteome (Ziv et al. 2008) strongly suggesting that this chaperonin was maternally

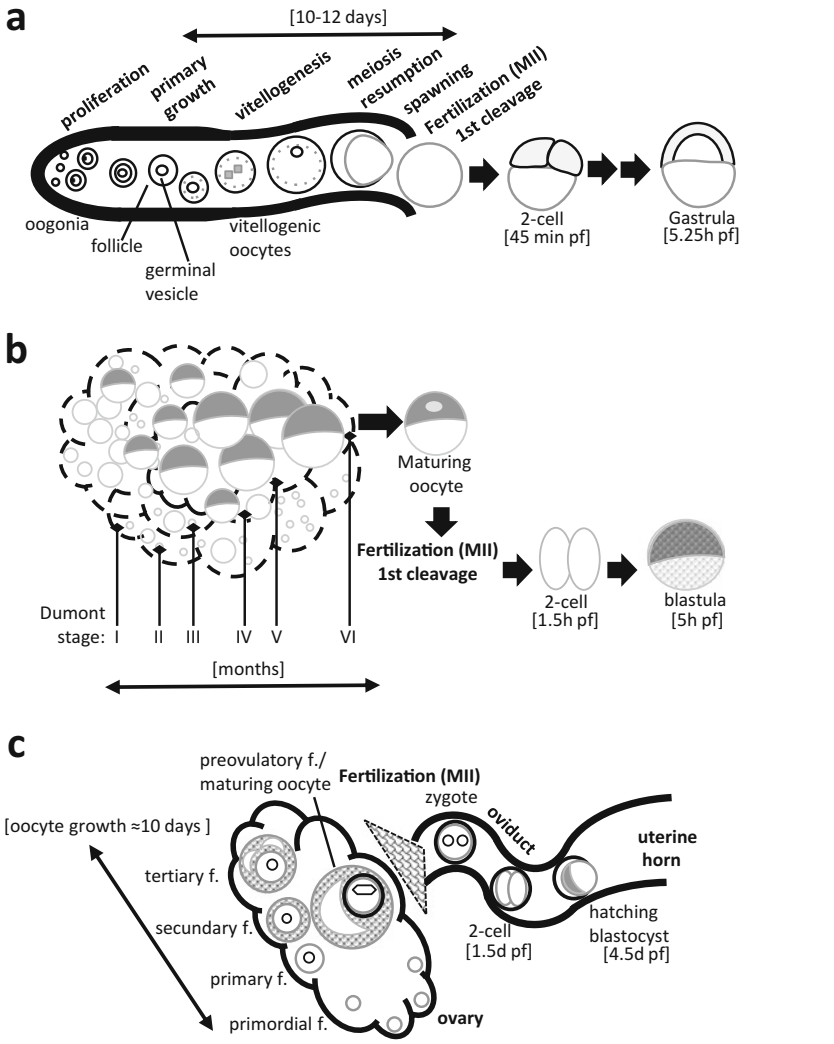

Fig. 3 Schematic illustration of oogenesis and early development in vertebrate experimental organisms (*Danio rerio*, *Xenopus laevis*, *Mus musculus*). (**a**) *D. rerio* oogenesis includes several stages: proliferation, pre-vitellogenic, and vitellogenic before resumption of meiosis. Mid-blastula transition is reached at 2.75 h post-fertilization. (**b**) *X. laevis* oogenesis is characterized by an important growth phase (vitellogenesis) lasting several months. After fertilization, rapid cleavages are under maternal control and mid-blastula transition occurs 5 h after fertilization. (**c**) *M. musculus* oogenesis depends on a defined stock of primordial oocytes, which undertake a rather limited growth phase before ovulation. Maternal to zygotic transition is accomplished by the 2-cell stage (about 20 h pf). Early development is slow in comparison to the other species: 4.5 days pf, the blastocyst is preparing its implantation in uterine wall. See text for additional information and references. pf: post-fertilization

Table 2 HS-related gene expression in zebrafish oocytes and early embryos

Transcriptome information (Aanes et al. 2011; Harvey et al. 2013; Rauwerda et al. 2016)	
DNAJ/HSP40 families:	
• No maternal transcripts—MII oocytes	*dnajb4, 5, 13, 14* *dnajc5b, 16, 22*
• Highest level of maternal transcripts (10)—MII oocytes	*dnaja2* *dnajb1b, 11, 12a* *dnajc1, 5gb, 6, 9, 11, 15, 18*
• Only maternal transcripts—MII oocytes	*dnajb1a, 2* *dnajc5gb, 30*
• Specific ↓ from 2-cell to 64-cell/MBT	*dnaja2* *dnajb1b, 6a, 11,12b* *dnajc2, 5ab, 5ga, 7, 8, 9, 11, 17, 18, 24, 28,*
• Specific ↑ from 2-cell to 64-cell/MBT	*dnaja1l, 3a, 3b, a4,* *dnajb4, 5, 6b, 9, 13, 14* *dnajc3, 4, 5aa, 6, 10,15, 19, 21, 22, 27,*
Small HSPs (HSPB) family:	
• High level—2-cell stage	*hspb1, 11*
• Level ≈ from 2-cell to 64-cell/MBT	*hspb11*
• Specific ↑ from 2-cell to 64-cell/MBT	*hspb1*
HSP70 (HSPA) family:	
• High level—MII oocyte & 2-cell stage	*hspa5, 8, 9, 14*
• Level ≈ from 2-cell to 64-cell/MBT	*hsp70.1, hspa12a, 12b, 8, 9*
• Specific ↓ from 2-cell to 64-cell/MBT	*hsp70.3, hspa8l*
• High level during preimplantation	*hspa8, 9*
HSP70 (HSPH) family:	
• Level ≈ from 2-cell to 64-cell/MBT	*hsph1*
• Specific ↓ from 2-cell to 64-cell/MBT	*hsph2a (hspa4a)*
• Specific ↑ from 2-cell to 64-cell/MBT	*hsph2b (hspa4b)*
HSP90 (HSPC) family:	
• High level—MII oocyte & 2-cell stage	*hsp90ab1, b1*
• Level ≈ from 2-cell to 64-cell/MBT	*hsp90aa1.2, ab1, b1*
• Specific ↓ from 2-cell to 64-cell/MBT	*hsp90aa1.1*
Proteome information (Knoll-Gellida et al. 2006; Ziv et al. 2008)	
DNAJ/HSP40 families:	
• Detected in oocyte	Dnajc3
HSP70 (HSPA) family:	
• Detected in oocyte	Hsp70.2, Hspa8, 9, 14
• Highest among Hspa family in oocyte	Hspa8
HSP90 (HSPC) family:	
• Detected in oocyte	Hsp90ab1, b1
• Highest among Hspc family in oocyte	Hsp90b1
Chaperonins:	
• Detected in oocyte	Hspe1

supplied. While transcript level could be differently measured due to the technical procedures and/or to variable level in polyadenylation, it should be also noted that Rauwerda et al. (2016) revealed some specific and relatively high variation of expression when comparing oocyte expression between five females. The most variable ones among HS-related genes were: *hsp70.1*, *hsp70.2*, *hspa14* and *dnajc3b*, *dnajc5gb*, *dnajc9*, *dnajc10*, *dnajc21*, and *dnajc24* (Rauwerda et al. 2016). It would be interesting to know whether those different levels are a specific feature for each female and would reproduce in their subsequent clutches or not.

From those expression studies, it was clear that *hsf1* and *hsf2* transcripts were present in the mature eggs as well as *hsbp1*, which is known as a negative interactor of HSF1 (Satyal et al. 1998). *Hsf1* mRNA level appeared relatively stable during early development while *hsf2* was significantly increasing (Harvey et al. 2013). The relationship between *hsf1*, *Hsbp1*, and *hsp* gene expression was shown in another study using morpholino (MO) experiments: 12 h pf embryos (segmentation stage, organogenesis) exhibited a dramatically reduced level of *hsp* transcripts (*hsp70*, *hsp90a*, *hsp110* when injected with *hsf1* MO and a marked increase in case of the *hspb1* MO (Eroglu et al. 2014). Nevertheless, it is difficult to know whether the subsequent phenotype features were due to Hsf1 perturbed function as Hsbp1 can interact with other transcription factors (Eroglu et al. 2014).

In zebrafish, loss-of-function experiments were abundantly performed using MO, but recently some concerns were raised about the poor correlation existing between MO induced and mutant phenotypes (Kok et al. 2015; Schulte-Merker and Stainier 2014). Therefore, it is important to carefully assess methodological aspects and results. This is also the reason why initiatives such as genome-wide screens for mutations in protein coding genes is expected to provide more conclusive data and ultimately improve our understanding of gene function (Kettleborough et al. 2013). Numerous HS-related genes have been already mutated by this consortium, but so far only *hsp90ab1* mutants were analyzed and classified as normal.

Even if numerous zebrafish transgenic lines contain some fragments of heat shock promoter, the classical heat shock response during oogenesis and very early development remains poorly described. HSR is expected to rely on zygotic transcription and therefore to be active after MBT (3.5 h pf). Most of the studies focused on later stages and therefore are beyond the scope of this chapter [reviewed in Rupik et al. (2011)].

3.2 Xenopus laevis/Xenopus tropicalis

X. laevis, the African clawed frog, is known for its large eggs (1 mm) that are well suited to biochemical studies and microinjection. Furthermore, oocytes produced in large numbers can be collected at various stages of oogenesis providing an easy source of material to investigate the expression and function of maternal factors to be stored in fully grown oocytes (Fig. 3b). Unfortunately, *X. laevis* has not performed well as a genetic model in comparison with the other animals listed in

this chapter because of its allotetraploid genome. A better knowledge of the genome and improvement in genome engineering can ameliorate the usefulness of amphibian models in particular with the diploid *X. tropicalis* (see Xenbase, Karpinka et al. 2015).

Regarding HS-related genes, the *Xenopus* genome contains several *hsfs* (*hsf*-1, 2, 2.2, 3, 4—*X. tropicalis*, −5), similar *hsp* (*hspa*/*hsp70* ; *hspb*—small *Hsps*, etc.), and *dnaj* (a, b, c = about 40 genes) families as those described in mammals (Kampinga et al. 2009). Nevertheless, in *Xenopus* and other lower vertebrates, the small *Hsp* group includes members with no mammalian equivalents: the acidic *hsp30* family (Heikkila 2016; Rupik et al. 2011).

After *D. melanogaster*, this experimental organism was instrumental in defining fundamental aspects of the HSR: evolutionary conservation, HSE sequence, Hsf binding activity, and nuclear localization as reviewed previously (Heikkila et al. 2007). In particular, Gordon et al. (1997) had noticed a constitutive Hsf DNA binding activity in oocytes from early stages (I, II), which disappeared as oocytes started to grow (Gordon et al. 1997). The constitutive complex was distinct from the stress induced one, which could be maintained all along development. In parallel with Hsf DNA binding activity, this study showed that the amount of *Hsp70* mRNA was high in early stage oocytes and decreased at later stages of oogenesis. This work suggested a potential maternal role for Hsf1, which could trigger the accumulation of *hsp* transcripts but also regulate non-*hsp* mRNAs as this could be speculated based on other studies carried out with mammals (Le Masson et al. 2011; Mendillo et al. 2012) (see 3.3). Unfortunately, to the best of our knowledge, there was no further investigation about those findings. Recent expression data showed that the level of *hsf1* transcripts was about 2 fold higher than *hsf2* transcripts, but a second gene *hsf2.2* found in amphibian and reptile genomes surprisingly exhibited abundant maternal transcripts (*hsf2.2*/*hsf1* = about fourfold) (Owens et al. 2016). Hsf2.2 is characterized by a conserved DNA binding domain, sharing 73% similarity with human HSF2. From this study about the developmental kinetics of gene expression, another interesting piece of information showed that both *hsf2* and *hsf2.2* mRNAs exhibited a peak at stage 9—blastula corresponding to transcriptionally active embryos. This could indicate a specific regulation of those transcription factors. This also raises the question of the respective role of Hsf1, Hsf2, and Hsf2.2 at the beginning of embryonic development in these organisms.

Studies on the developmental expression of *hsp* genes in oocytes and embryos were previously compiled by Rupik et al. (2011). Interrogating the transcriptomic data published by Owens and colleagues provides a series of interesting observations (Owens et al. 2016). Only *hspa8* from *Hspa*/*Hsp70* family was found to be increasingly expressed along development with a low level of maternal transcripts. RNA expression of small *hsps* from the *hspb* group was low in early embryos. In contrast some members of the *hsp90* family were characterized by a relatively abundant maternal stock (*hsp90ab1* > *hsp90b1*). Only few members of the *dnaj* families displayed a high level of transcripts per early embryo (Stage 1) meaning that they were provided by the oocyte so that they could be listed as a maternal supply: *dnaja2*, *dnajb6*, *dnajb9*, *dnajc9*, *dnajc19*. A series of HS-related proteins

could be also identified in the databases of recent proteomic analyses comparing various early stages from egg to late neurula stage [(stages 1-egg, 5–16-cell, 8-blastula, 11-gastrula, 13-neurulation, 22-neurula; (Sun et al. 2014)]: Dnaja1, 2, 4; Dnajb11, 14; Dnajc8, 9, 11, 17, 18, 25; Hsc70; Hsp70; Hspa4, 5, 9b; Hsp90ab1; Hsp105; Hspd1. From this limited set of Hsps, only three displayed some changes over the studied stages, while the others remained stable. Hsp70 decreased from the egg stage while Hsp90ab1 and Hsp105 present from the egg stage appeared to increase from the blastula stage. When conducting blastomere comparison at 2, 8, 16, 32, and 50 cell embryos, the protein level for Hsps seemed to be homogenous between cells except two Hsp90: Hsp90ab1 and Hsp90b1 that exhibited significant variation between blastomeres at the 50-cell stage (Sun et al. 2016). Those proteomic studies performed under normal developmental conditions are appealing as they demonstrate the feasibility of dissecting protein expression during development and eventually during stress responses. As often, transcriptomic and proteomic data were not fully overlapping either because of technical differences or because of specific control of gene expression at the post-transcriptional level. At least it could be speculated that there was a well-defined and distinct regulation of the maternally provided chaperones either as transcripts or proteins.

The functional role of Hsps in oocytes and during early development remains much less understood. We will describe two studies that highlighted the potential importance of HS-related proteins in oocytes. The first one provided a not so surprizing link between Hsp90 and a kinase involved in the cell cycle. Xenopus oocytes/egg are extensively used to study cell cycle mechanisms during meiosis and mitosis. Yamamoto and collaborators identified Greatwall (Gwl) kinase as a client of Hsp90 (Yamamoto et al. 2014). Gwl is an important kinase, which takes part in a potential activation loop of the Mitosis Promoting Factor (MPF). Gwl is phosphorylated by MPF and inhibits a phosphatase acting on the MPF substrates. Gwl is required for oocyte maturation. Therefore, when the stability of Gwl is reduced to 30 min in egg extracts by inhibiting Hsp90 with geldanamycin, this could compromise the G2/M transition and M maintenance in maturing oocytes. The second study brought some insights into the functions of Dnaj, which remained poorly understood for most of the members of this HS-related family. Following maturation, eggs are ready to be fertilized and sperm–egg fusion is known to induce cortical granules exocytosis, an important phenomenon preventing polyspermy. To study in detail the molecular mechanisms, immature oocytes are a valuable experimental system where it is possible to chemically induce cortical granule exocytosis. Smith and colleagues showed that overexpression of Dnajc5b, also named CSP for cysteine string protein, inhibited this chemically induced exocytosis (Smith et al. 2005). CSPs are a class of proteins associated to regulated secretory mechanisms, e.g., synaptic vesicles. Dnaj protein are characterized by a J-domain, which interacts with proteins from the Hsp70 family. Their study reviewed in Heikkila et al. (2007) revealed that Dnajc5b by this interaction sequestered Hsc70 preventing the chaperone to participate in the exocytosis mechanism.

*3.3 Mammals (*Mus musculus*)*

Cellular and molecular mechanisms involved in mammalian reproduction (gametogenesis, early embryonic development) is of direct interest for human patients who look for highly sophisticated manipulations in the context of medically assisted reproduction. In contrast to our species whose oocyte supply for experimental investigation remains scarce (one oocyte per cycle in absence of ovarian stimulation), mouse can produce multiple oocytes and can be genetically manipulated, two significant experimental advantages. At the end of the intraovarian growth phase, mouse eggs, which remain relatively small (80 μm diameter), are ovulated at Metaphase II (MII) stage. The transition between maternal control of development to zygotic control occurs relatively early at the 2-cell stage even if the timing is slow in comparison to the other experimental species described in this chapter (Fig. 3c) (Tadros and Lipshitz 2009).

Previous reviews reported heat shock gene-related expression and functions during gametogenesis and embryonic development in this experimental mammalian species (Abane and Mezger 2010; Christians et al. 2003; Christians and Benjamin 2005; Rupik et al. 2011). In this chapter, we searched several recent transcriptomic and proteomic databases to further establish the catalog of HS-related genes expressed during oogenesis and early embryonic development (Abe et al. 2015; Veselovska et al. 2015; Zhang et al. 2009).

The mammalian genome harbors up to six Hsf-related genes but Hsf1 and *2* are considered as the main HS-related factors (Akerfelt et al. 2010). Soon after their cloning, Hsf1 was found to be highly expressed in the MII (metaphase II) oocyte while Hsf2 transcripts accumulated later during preimplantation development (Christians et al. 1997). This was confirmed by transcriptomic data (Le Masson et al. 2011; Abe et al. 2015). Analysis of the Hsf1 and Hsf2 transcript levels during oogenesis revealed that Hsf2 transcripts could be as abundant as Hsf1 in non growing oocytes and then even more represented than Hsf1 during oocyte growth (Veselovska et al. 2015). To know more about the HSF1 and HSF2 protein levels would be interesting, but it remains limited to indirect evidence based on DNA binding activity showing that HSF1 activity could be detected at all stages from mature oocytes to blastocyst while HSF2-dependent activity was identified at the blastocyst stage (Mezger et al. 1994a, b).

In order to define a more precise profile for HS-related gene expression during oogenesis and in the early embryo, we interrogated transcriptomic and proteomic studies. They were not initially designed for that purpose and therefore did not necessarily provide exhaustive information, i.e., they did not contain data for all the HS-related genes. Thus, no conclusion should be drawn about missing/non-listed genes. A qualitative, commentated analysis of the data is summarized in Table 3. The main features were (1) a distinct maternal set of several Dnaj family members, (2) a high maternal supply of Hsp90 family members, (3) a low or nonexistent contribution from the small Hsp family.

Table 3 HS-related gene expression in mouse oocytes and early embryos

Transcriptome information (Abe et al. 2015; Veselovska et al. 2015)	
DNAJ/HSP40 families:	
• Specific upregulation—oocyte growth	Dnaja1 Dnajb9, 11, 12, 14 Dnajc1, 3, 5, 5b, 7, 8, 9, 11, 13, 14, 18, 19, 21
• Specific upregulation—GV/fully grown oocyte	Dnaja4 Dnajb13 Dnajc4, 5b, 17, 22, 30
• High level of maternal transcripts—GV/fully grown oocytes	Dnaja2, 4 Dnajb1, 2, 4, 6, 12 Dnajc3, 5, 8, 11, 16
• High level of maternal transcripts—MII oocytes	Dnajc3, 5, 17
• High level during preimplantation	Dnajc5, 13
Small HSPs (HSPB) family:	
• Specific upregulation—oocyte growth	Hspb11
• Highest level of maternal transcripts within family	Hspb11
HSP70 (HSPA) family:	
• Specific upregulation—oocyte growth	Hspa1a,5 (Bip), 8, 9, 12a
• High level of maternal transcripts—GV/fully grown oocytes	Hspa8, 9
• Maintained level during preimplantation	Hspa5, 8, 9
• High level during preimplantation	Hspa8
HSP70 (HSPH) family:	
• Specific upregulation—oocyte growth	Hsph1, 2 (hspa4)
HSP90 (HSPC) family:	
• Specific upregulation—oocyte growth	Hsp90aa1
• High level of maternal transcripts—GV/fully grown oocytes	Hsp90aa1, ab1, b1
• High level during preimplantation	Hsp90aa1
Proteome information (Schwarzer et al. 2014; Wang et al. 2010; Zhang et al. 2009)	
DNAJ/HSP40 families:	
• Highest level in GV/fully grown oocyte	DNAJB6, 5
• Higher level in oocyte > zygote	DNAJA1, 2, 3, 4 DNAJB1 DNAJC3, 5, 9, 11, 13
• Lower level in GV oocyte < zygote	DNAJB4, 11 DNAJC7, 19
• Stable level in MII oocytes through female aging	DNAJA1, 2, 3, 4 DNAJB4, 6, 11 DNAJC7, 8, 9, 11, 13
• Fourfold increase in oldest female	DNAJB4
Small HSPs (HSPB) family:	
• Stable level in MII oocytes through female aging	HSPB1

(continued)

Table 3 (continued)

HSP70 (HSPA) family:	
• Stable level in MII oocytes through female aging	HSPA5, 8, 9, 14
• Highest among HS-related gene in MII oocyte	HSPA5, 8, 9
• Specific ↑ for MII oocyte and ↓ in zygote	HSPA1A,1B, 2, 5, 8, 9
HSP70 (HSPH) family:	
• Stable level in MII oocytes through female aging	HSPH1
• Highest among HS-related gene in MII oocyte	HSPH2 (HSPA4)
• Specific ↑ for MII oocyte and ↓ in zygote	HSPH1, 2, 3
HSP90 (HSPC) family:	
• Stable level in MII oocytes through female aging	HSP90AA1, AB1, B1
• Highest among HS-related gene in MII oocyte	HSP90AA1, AB1, B1
• Specific ↑ for MII oocyte and ↓ in zygote	HSP90AA1, AB1, B1
Chaperonins:	
• Stable level in MII oocytes through female aging	HSPD1, HSPE1
• Highest among HS-related gene in MII oocyte	HSPD1

HSF1 constitutive and conditional knockout mice were generated independently in several laboratories (Fig. 2c), and the absence of HSF1 in oocytes consistently resulted in female infertility. The situation was less clear regarding HSF2 as some lines induced a reduced fertility while others did not seem to significantly impact female reproduction (Abane and Mezger 2010; Christians and Benjamin 2005). It could be hypothesized that variable expressivity of the reproductive phenotype could reflect *Hsf2* expression during oogenesis (Veselovska et al. 2015).

HSF1 loss-of-function differently impacted Hsp expression: *Hsp70.1* (*Hspa1b*) > *Hsp25* (*Hspb1*) > *Hsp90alpha* (*Hsp90aa1*) > *Hsp105* (*Hsph1*) while no effect could be detected on *Hsp90beta* and *Hsp60*. As a consequence, the $Hsf1^{-/-}$ oocyte, which should have been loaded with a large amount of Hsp90alpha, was strongly depleted and this reduced the amount of Hsp90alpha in addition to other maternal products that could contribute to a series of meiotic anomalies: G2/M delay, Metaphase I block, a large polar body due to the absence of asymmetric cell division (Metchat et al. 2009; reviewed in Abane and Mezger 2010). HSF1 function can be modified through protein–protein interactions, which involves versatile molecular complexes made of several HSPs but also non-HSP protein as Hsbp1 (Satyal et al. 1998). In mammals, *Hsbp1* gene targeting provoked an early embryonic death, just at the transition between free and early implanted blastocyst (around 5d pf), probably when the maternal supply had to be replaced by the embryonic one (Eroglu et al. 2014). As HSF1, Hsbp1 is a maternal factor but because of this lethal phenotype, a conditional knockout in oocytes should be generated to fully understand its role. Furthermore, Hsbp1 was shown to interact with other transcription factors, so it must be determined whether HSF1 deregulation was involved in blastocyst degeneration in *Hspbp1* mutant mice (Eroglu et al. 2014).

Table 4 Effect of HSF1, HSF2 loss-of-function on HS-related gene expression (Le Masson et al. 2011)

		Gene expression after loss-of-function	
Genes listed on microarray:		HSF1[a]	HSF2[a]
NON significant level in WT oocyte			
Hspa	1a, 1b, 1 l, 12a, 14	N/A	
Hspb	1, 2, 3, 7, 8, 9		
Hspc	–		
Dnaja	1		
Dnajb	3, 5, 7, 8, 9, 12, 13, 14		
Dnajc	1, 2, 4, 5, 6, 12, 18, 19, 22, 24, 27, 28, 30		
Significant level of expression in WT oocyte			
Hspa	2 (Hsp70.2), 4, 4 l, 5, 8 (Hsc70), 9	2↓, 8↓	–
Hspb	11	11↓	–
Hspc	1 (Hsp90aa1), 3 (Hsp90ab1), 4 (Hsp90b1),	1↓[b]	≈
Hsps	Hspe1, Hspd1, Hsph1	≈	–
Dnaja	2, 3, 4	4↓	4↓
Dnajb	1, 2, 4, 6, 11	2↓	≈
Dnajc	1, 3, 5b, 8, 9, 11, 14	5b↑	–

[a]Minimum of twofold change ↑ or ↓. No change: ≈
[b]Previously analyzed in Metchat et al. (2009)

Even if *Hsf2*$^{-/-}$ females we studied remained fertile (Le Masson et al. 2011; McMillan et al. 2002), the impact of HSF1 and HSF2 loss was analyzed and compared to better understand the respective contribution of both factors on the immature (GV) oocyte transcriptome (Table 4). Taking into account Hsp genes whose transcripts were significantly detected on the microarray, this study was in agreement with more recent ones and showed that the fully grown oocyte chaperome lacked most of the Hspb family members (or small heat shock protein genes) and expressed a distinct selection of Dnaj genes (or HSP40 families) (Le Masson et al. 2011; Abe et al. 2015; Veselovska et al. 2015). More limited was the number of genes whose transcript level was affected by the absence of HSF1. Of interest, some rather unknown, neglected genes relied on HSF1 such as Hspb11, a small heat shock protein, which could be involved in mitochondria organization and cancer (Turi et al. 2015). Only one co-chaperone, Dnajc5b, was significantly upregulated in absence of HSF1, suggesting a repressive role for the transcription factor. This gene was mentioned above because of its role in cortical granules exocytosis in *Xenopus* in association with Hsc70 (Hspa8) (Smith et al. 2005). It is intriguing to notice that HSF1-deficient oocytes exhibited abnormal cortical granule exocytosis so that some functional link could be established with this co-chaperone (Bierkamp et al. 2010). Loss of HSF2 altered the expression of 959 genes in oocyte transcriptome and about 24% were shared with HSF1 (Le Masson et al. 2011). Our analysis focusing on HS-related genes revealed that only one gene was significantly expressed in oocytes and affected by both HSF1 and HSF2 loss: Dnaja4. Very little is known about this gene during oocyte

development besides its occurrence in several transcriptomic analyses, which reported that this gene is expressed as a maternal factor during oocyte growth up to fertilization and then is degraded as the zygotic genome activation occurs.

To further identify which HS-related gene is required for either oocyte or early embryo development, the mouse genome informatics (MGI) database was searched by genes and by phenotypes. From the oogenesis-female gamete point of view, the results were disappointing as heat shock-related genes appeared to be mostly necessary for spermatogenesis but not for oogenesis. Besides the already discussed HSFs, one *Hsp40* (*Dnaja3*, *Tid1*), *Hsp60*, *Hspa5*/*Bip*/*Grp78* mutants (see chapter "Roles of Grp78 in Female Mammalian Reproduction") affected early development provoking embryonic death around implantation (Christensen et al. 2010; Lo et al. 2004; Luo et al. 2006). *Hsp90b1* knockout mice seemed to survive a little bit longer up to 10d pf (Mao et al. 2010). This suggests again that the maternal contribution was critical to maintain viability of the mutant embryos, which could not survive once the oocyte supply was exhausted. The altered mechanisms leading to embryonic lethality remain poorly understood. DNAJA3 binds Hsp70s through its J domain so its absence can impact a large part of the chaperone activity required in the embryo in contrast to the knock-out of a single Hsp70 gene. Furthermore, DNAJA3 is also a binding partner for numerous other proteins by which it can be involved in multiple functions, e.g., mitochondrion organization. HSP60 is also an important mitochondrial protein, which shares with DNAJA3 some interactors such as members of Hsp70 family. This short list should be soon expanded as an international consortium has undertaken the huge task to knock-down each gene and analyze the phenotype of the resulting mice (Dickinson et al. 2016). From their available list at the time of writing this paper, several additional members of the Hsp40 family had their KO phenotype described and two of them, *Dnajc8* and *Dnajc9*, appeared to be embryonic lethal around 9.5d pf while their maternal expression was noted in transcriptomic and proteomic databases (Veselovska et al. 2015; Wang et al. 2010).

As mentioned above, embryonic lethality of homozygous mutants often prevents further investigation, in particular regarding reproductive function. In the case of Hsp90b1, we had the opportunity to create an oocyte-specific knockout. Depleted oocytes looked rather normal with a slightly thinner zona pellucida. More importantly, those oocytes could not support the correct sequence of events required for the first mitosis post-fertilization. The main defect was the disorganization of the mitotic spindle, but the molecular mechanisms remain to be fully understood (Audouard et al. 2011).

Finally as in other species, the classical heat shock response (HSR) was shown to exist during oogenesis and disappear in fully grown oocytes as they become transcriptionally silent. However, in contrast to other species, the mouse HSR was progressively reestablished during preimplantation development (Christians et al. 1997). In this species, it was demonstrated that 2-cell stage embryos were fully transcriptionally active but only blastocysts were truly HSR competent (Bouniol et al. 1995; Christians et al. 1997).

4 Conclusion and Future Perspectives

In all species practicing either sexual reproduction or parthenogenesis, the content of fully grown oocytes or eggs ready to be fertilized plays a critical role to ensure proper embryonic development occurs after fertilization. Examples described in this chapter, which covered only a limited sample of experimental organisms, have confirmed the importance of several heat shock-related genes within this physiological context.

Considering the functional aspects related to the stress response, the data reported in this chapter have not solved several questions or apparent contradictions.

- How is HSF activity regulated during the classical heat shock response and during oogenesis? Oogenesis and early embryogenesis represent puzzling developmental stages during which the classical heat shock response disappears and remains silent before reappearing progressively at later embryonic stages.
- How to understand at the molecular level that on one hand, HSPs and HSFs, defined as actors of the stress response, are required during early development in absence of visible stress (i.e., in normal physiological conditions) while on the other hand during this same period of development, the classical heat shock (stress) response is nonfunctional making young embryos very sensible to various stresses? At least two overlapping explanations can be proposed even if they are not fully satisfying: first, oocytes and early embryos contain a sufficiently high level of HSP so that the chaperone function can be achieved to cope with moderate stress in absence of an inducible transcriptional activity, which can be considered costly; second, oocytes and early embryos must be "perfect" to achieve a successful development so that if they encounter cellular stress, and even if they could survive thanks to defense mechanisms, they would probably suffer some alterations that could affect the future organism. Therefore, under such conditions death is preferred to eliminate faulty offspring and protect the species (Rupik et al. 2011).

Even if comparison of the information available in the described species remains difficult due to scattered data and various experimental design or methodologies, there are some common features among the five species described here. HSF(1) is important for oogenesis and reproduction. HSPA8, members of HSP90, family, are commonly expressed at high levels in oocytes. In contrast, small HSPs are present during oogenesis in the invertebrate but are barely detectable in vertebrates. For now there is no explanation for this difference in small HSP requirements.

As for many other genes and/or biological system, new tools such as large transcriptomic/proteomic databases or easier ways to modify the genome are increasingly improving our knowledge of HS-related genes. From this chapter, it clearly appears that oocyte chaperome and HS-related transcription factors should receive more attention since they are essential for developmental success.

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The Unmysterious Roles of HSP90: Ovarian Pathology and Autoantibodies

Eusebio S. Pires

Abstract The heat shock proteins (HSPs) are a group of evolutionarily conserved proteins with important physiological functions, whose synthesis is enhanced by elevated temperature or other stresses. HSPs show high sequence homology between different species, from bacteria to humans. Despite the significant degree of evolutionary conservation, HSPs are highly immunogenic. Of the several HSPs, HSP90 is an abundant, constitutively expressed chaperone constituting around 1–2% of total cellular protein under non-stress conditions. This protein from even the most distantly related eukaryotes has 50% amino acid identity, and all have more than 40% identity with the *Escherichia coli* protein. They are immunodominant antigens for many common microbes, and thus their epitopes are recognized by the immune system. As HSPs are overexpressed at sites of acute and chronic inflammation, individuals are likely to be sensitized during the course of a microbial infection encountered during life. This chapter considers the evidence of a role for HSP90 in autoimmune ovarian failure, where autoantibodies to it have been observed in patients, and has been correlated to infertility.

1 Introduction

As I studied Immunology right from my days of graduation, I kept asking myself one of the most important questions in Immunology—how does the immune system distinguish between "*self*" and "*nonself*"? In a normal, healthy individual, the immune system is able to specifically eliminate unwanted, non-self, and potentially dangerous organisms without attacking its own tissues or cellular components. In some cases, however, this fine-tuning is disturbed leading to autoimmunity: the activation and proliferation of autoreactive lymphocytes or even to an autoimmune disease. In the case of organ-specific autoimmunity, the antibodies produced by the activated B-lymphocytes are directed to self-components expressed only in a

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specific tissue or type of cells. In the case of systemic autoimmunity, the autoantibodies are directed to various autoantigens, which are usually expressed in a wide variety of tissues and, at the cellular level, can be present in the nucleus, cytoplasm, or at the cell surface. Although many autoantigens are ubiquitously expressed, the autoimmune diseases in which they are autoantigenic are often limited.

2 Autoimmunity and Ovarian Autoimmunity

The human body can undergo an autoimmune attack, and autoimmunity of most organs has been reported leading to autoimmune disease which may or may not be life threatening. An increasing number of individuals throughout the world are affected by autoimmune disease, and a large and diverse group of disorders have been categorized by tissue injury or pathology (Lernmark 2001). In general, these diseases are associated with humoral or cell-mediated immune reactions against one or more of the body's own constituents, but it has been customary to divide autoimmune diseases into two categories: systemic and organ-specific. Over the past decades, the list of diseases associated with autoantibodies against tissues, cells, or specific autoantigens has grown enormously (Lernmark 2001). The classification of a disease as autoimmune has traditionally been based on the detection of autoantibodies that could be visualized reacting with an affected tissue or cell. Like all other organs, the reproductive tissues also undergo an autoimmune attack. Many scientists have contributed enormously towards this field of research and to date there have been several differences in opinions. The human ovary is a target of an autoimmune attack in various circumstances, including several organ-specific or systemic autoimmune diseases. Clinically, the ensuing ovarian dysfunction often results in premature ovarian failure (POF), but other pathologies involving the ovaries, such as unexplained infertility, polycystic ovary syndrome (PCOS), and endometriosis, have been associated with anti-ovarian autoimmunity (Luborsky 2002; Ahonen et al. 1987; Anasti 1998; Coulam et al. 1986). POF or premature menopause (or recently rechristened to primary ovarian insufficiency, POI) is a syndrome clinically defined by functional failure of the ovary before the age of 40 years. POF is a heterogeneous disorder with a multicausal pathogenesis, and chromosomal, genetic, enzymatic, iatrogenic, or infectious aberrations may all form the basis for the disappearance of ovarian follicles (Hoek et al. 1997). These aberrations may influence the ovary at any stage of life, including the prepubertal, pubertal, or reproductive stages. There is accumulating evidence that some cases of POF are due to a faulty recognition of self in the ovary by the immune system (Hoek et al. 1997). POF was defined by de Moraes-Ruehsen and Jones (de Moraes-Ruehsen and Jones 1967) as an unphysiological cessation of menses before the age of 40 year and after puberty (hence, in fact, secondary amenorrhea). Women with POF have a hypergonadotropic-hypoestrogenic hormone profile (Hoek et al. 1997). The involvement of autoimmunity has been most extensively studied in

POF. However, the etiological significance of autoimmunity in these pathologies still remains controversial.

3 Antigens Involved in Ovarian Autoimmunity

The diagnosis of an autoimmune mechanism in these pathologies has relied for a long time on the detection of anti-ovarian antibodies (AOA) (Barbarino et al. 2005; Betterle et al. 2002; Damewood et al. 1986). However, little is known about the molecular targets of the autoimmune effectors, and very few autoantigens have been formally identified. The specificity of the available tests has also been questioned (Fenichel et al. 1997; Novosad et al. 2003). Our group developed a novel blocking protocol (Pires et al. 2006) which appreciably reduced the nonspecific reactivity. Using this specific and sensitive test, we have been able to identify a number of specific molecular and cellular targets (Pires et al. 2007) and found that AOA testing has clinical significance (Pires et al. 2011a). From the large number of samples screened, we found that the oocyte is the major cell that is being targeted (Pires et al. 2007). It was also seen that a large number of AOA positive samples reacted with a 90 kDa protein, indicating that this protein (designated EP90) was an immunodominant antigenic target (Pires et al. 2007). This well conserved acidic protein was oocyte specific, serine–threonine phosphorylated, non-glycosylated, and expressed in the day 0 old rat ovary and onwards (Pires and Khole 2009a). LC/MS and MS/MS analysis of this EP90 protein revealed its identity to be human heat shock protein 90 beta (HSP90β) (Pires and Khole 2009a). The identity of EP90 was then reconfirmed using the patient's sera in two sets of experiments. First, most of the EP90 reactive sera of POF patients were seen to react with a recombinant HSP90 protein. Second, a monoclonal antibody to HSP90 showed reactivity with the partially purified EP90 protein. Our data, therefore, suggested that HSP90β could be a major autoantigen of self-reactive antibodies in the study group (Pires and Khole 2009a, b; Pires 2010; Pires et al. 2011a, b; Khole et al. 2012). Antibodies to HSP90 in a female mouse model were generated by active immunization with an immunodominant peptide of HSP90. There was a significant drop in the fertility index due to an increase in pre- and post-implantation loss, associated with an increased incidence of degenerated eggs and embryos. The ovaries showed an increase in the number of empty and degenerated follicles and extensive granulosa cell deaths, which was reflected by the decrease in the levels of Nobox and Gja1 gene expression (Choudhury and Khole 2013). In addition to HSP90 protein as a target, patients are also likely to have several other antigens including P450 side chain cleavage (SCC) enzyme, 17-hydroxylase, α-enolase (Forges et al. 2004; Sundblad et al. 2006), and also different molecular targets (Pires et al. 2007) including reports of alpha actinin, HSP70, and beta actin as targets (Mande et al. 2011).

4 Heat Shock Proteins (HSPs)

HSPs or chaperonins, as they were previously called, are a group of evolutionary conserved proteins that show high sequence homology between different species, from bacteria to humans (Morimoto 1993). They are classified based on molecular size, sequence similarities, and location within the cell and function. Despite the significant degree of evolutionary conservation (Sreedhar et al. 2004), HSPs are highly immunogenic (Calvert et al. 2003). It has been postulated that they could activate antigen-presenting cells, serving as a danger signal to the immune system (Gallucci and Matzinger 2001).

5 Heat Shock Protein-90 (HSP90) in Ovarian Biology, Localization, and Function

Of the several HSPs, HSP90 is an abundant, constitutively expressed chaperone constituting around 1–2% of total cellular protein under non-stress conditions (Falsone et al. 2005; Lindquist and Craig 1988). This protein from even the most distantly related eukaryotes has 50% amino acid identity, and all have more than 40% identity with the *Escherichia coli* protein (Bardwell and Craig 1987). There are two major cytoplasmic isoforms of HSP90: HSP90α and HSP90β, which possibly arose by gene duplication roughly 500 million years ago (Gupta 1995). Sequence similarities between the α and the β forms are 93.4% using the EBI tool: EMBOSS pairwise alignment algorithm. Heat-shock protein-90 is mainly a constitutive dimer (α α or β β); however, monomers (α or β), heterodimers (α β), and higher oligomers of both isoforms also exist (Sreedhar et al. 2004). An important difference is that the α form readily dimerizes, whereas the β form does so with much less efficiency (Sreedhar et al. 2004). Expression of HSP90α is lower compared with HSP90β in most cells, and HSP90α is highly inducible in contrast to HSP90β, whose expression is thought to be constitutive (Hilscher et al. 1974; Gruppi et al. 1991). The isoform specificity is not restricted only to the biochemical level, but extends to the functional role of HSP90 in cell differentiation and development. On the one hand, HSP90α has been shown to play a regulatory role in muscle cell differentiation of zebrafish (Lele et al. 1999), while on the other hand it is shown to inhibit cellular differentiation of embryonal carcinoma cells to trophectoderm (Voss et al. 2000). Studies have shown that HSP90β plays a major role in trophoblast differentiation, and HSP90β-deficient homozygous mice with normal expression of HSP90α fail to differentiate to form placental labyrinths (Voss et al. 2000). Expression of HSP90β is observed throughout the germ cell lineage from very early stages of development to adult oocytes and spermatocytes (Ohsako et al. 1995). Studies have suggested that HSP90β may be required for early

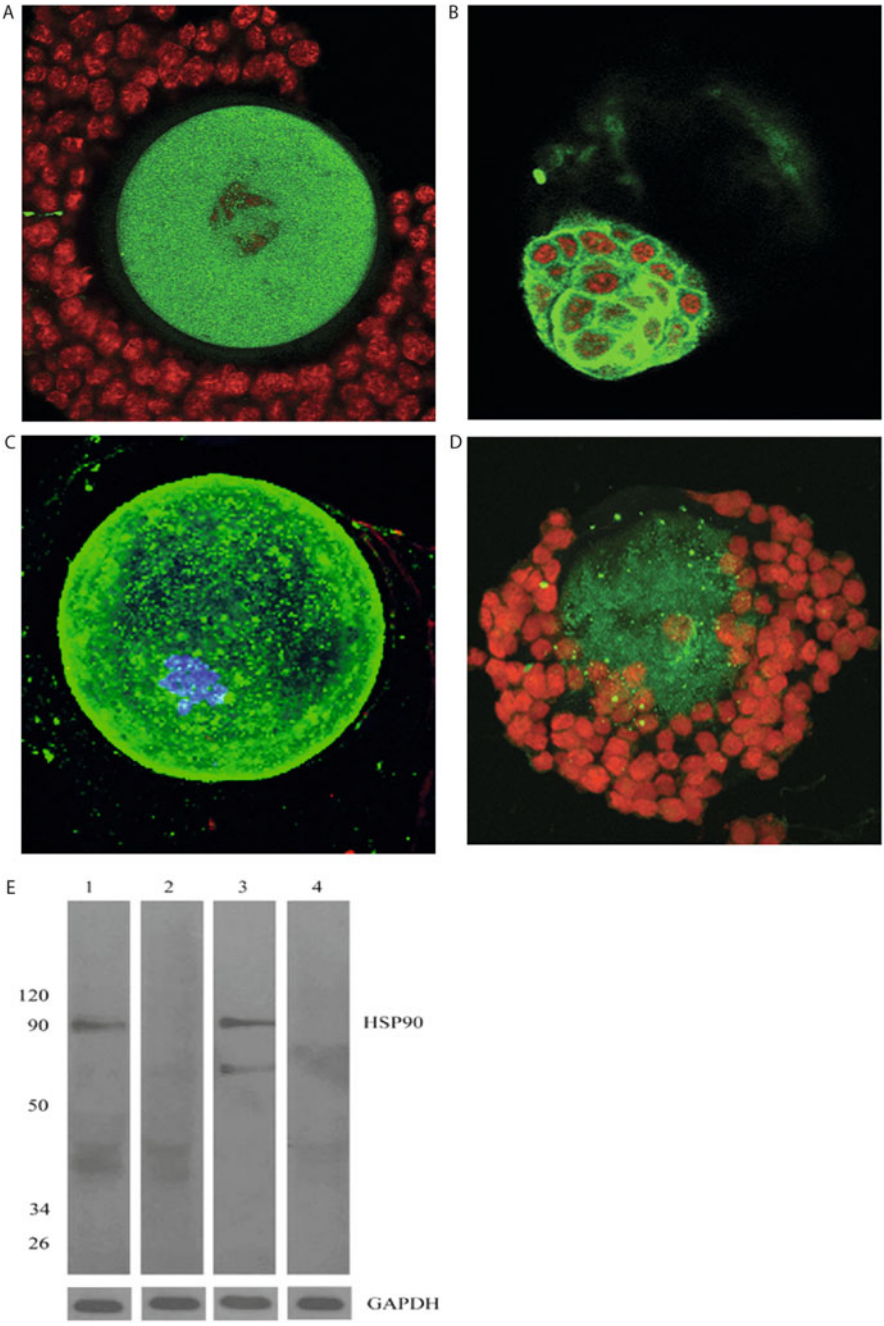

Fig. 1 HSP90 expression in the oocyte. Confocal images of HSP90 expression (*green staining*) in mouse embryogenesis showing immunostaining in the germinal vesicle breakdown oocyte (GVBD; **a**) and in the cells of a blastocyst (**b**). Nuclei were counterstained with propidium iodide as seen in *red*. Indirect immunofluorescence studies using anti-EP6 HSP90 peptide polyclonal antibodies showed surface expression (*green stain*) in an ovulated mouse oocyte (**c**) while the

embryonic development. Experiments from the lab definitively indicated that HSP90β is expressed in the ovary and abundantly in the oocytes and the early embryo (Pires and Khole 2009a). The experiment involved indirect immunofluorescence where a high titer anti-HSP90β positive patient sera was used to stain mouse oocytes and embryos. The sera immunostained the oocyte (green stain) within a germinal vesicle breakdown (GVBD) follicle as well as other stages of embryogenesis till the blastocyst stage, staining the cells of the inner cell mass as well as the trophectoderm (Fig. 1a, b). An anti-EP6 peptide-specific rabbit polyclonal antibody raised in the lab (Pires et al. 2011a) similarly stained the mouse oocytes (Fig. 1c) and embryos while a commercially available HSP90 antibody also stained the oocytes (Fig. 1d). It was interesting to note that the granulosa cells or the cumulus mass were immunonegative. In the same figure, a Western blot analysis was done using ovarian protein extracts and immunoprobed with either commercially available HSP90α or HSP90β polyclonal antibodies. The figure clearly shows that the β form was ovary specific (Fig. 1e, lane 1) while the α form was present in the testes (Fig. 1e, lane 3) and was not in the ovary (Fig. 1e, lane 2).

6 HSP90 Autoantibodies and Ovarian Pathology

HSPs are highly evolutionarily and phylogenetically conserved. They are immunodominant antigens for many common microbes and therefore their epitopes are recognized by the immune system (Van Eden et al. 2002). As HSPs are overexpressed at sites of acute and chronic inflammation (Van Eden 1999), many infertile couples are likely to be sensitized during the course of a microbial infection which they are likely to encounter during life. In view of this, it could be proposed that as a result of prolonged or repeated asymptomatic chronic infections early in the life of these infertile women, they could have developed anti-HSP90 antibodies systemically. These antibodies could thus target the ovarian antigens leading to ovarian failure. This may be relevant to human reproduction, since many couples with fertility problems have had a previously undetected genital tract infection (Witkin et al. 1994). In general, HSPs are among the first proteins produced during embryogenesis (Bensaude and Morange 1983). As a consequence of this, pregnancy outcome may be affected since the constitutive forms of HSP90 (and also

Fig. 1 (continued) nucleus was stained with DAPI (*blue*). Similar immunoreactivity with a commercially available monoclonal antibody to HSP90 was seen in the ooplasm of a mouse oocyte (**d**). No staining in the cumulus granulosa cells was observed. Western blot analysis (**e**) depicted the major isoform in total human ovarian extracts to be the beta isoform of HSP90 as seen at the 90 kDa loci when probed with a commercially available HSP90β antibody (lane 1). No immunoreactivity was seen with a commercially available HSP90α antibody (lane 2). Mouse testicular extracts were used as a positive control for the HSP90α antibody (lane 3). "No primary/secondary alone" antibodies served as negative control (lane 4). Antibody to GAPDH served as loading control to ensure equal amounts of protein per lane

HSP70) are both known to be expressed at high levels during preimplantation mouse embryo development (Loones et al. 1997).

The matured MII egg is transcriptionally an inactive cell and as such is a storehouse of maternal proteins and mRNA required for fertilization and initiation of zygotic development (Calvert et al. 2003). Using the approach of 2D Proteomics and Tandem Mass Spectrometry, this group reported that among the hundreds of proteins that are expressed by the mammalian oocyte, most of them are molecular chaperones and HSPs. Involvement of anti-HSP90 antibodies in pathogenesis of several diseases has been reported by several researchers in various other disease conditions. Faulds et al. (1995) have shown the presence of the antibodies in systemic lupus erythematosus, Hayem et al. (1999) have shown these antibodies in serum of patients with rheumatoid arthritis, Trieb et al. (2000) discussed the presence of this antibody in osteocarcinoma patients, and Vidal et al. (2004) have shown the presence of HSP90 antibodies in women with ovarian cancers. My group were the first to report the presence of anti-HSP90 antibodies in women with infertility (Pires and Khole 2009a). In our study, our data clearly showed that sera from 59 out of the 79 patients who were 90-kDa positive reacted to HSP90 by dot blot as well as ELISA.

The involvement of multiple antigenic targets, the high prevalence of anti-HSP90 antibodies, and the broad gamut of immunological disorders in which anti-HSP90 antibodies are found support the proposal that anti-HSP90 antibodies could be present in patients with a putative defect in immunoregulation. To establish importance of AOA testing in infertile women, a clinical reproductive outcome comparative study was conducted between two groups of women undergoing IVF-ET (Pires et al. 2011b). Group 1 consisted of women who tested positive for AOA, put on corticosteroid therapy, reverted to AOA negative, and then taken up for IVF-ET. Group 2 was seronegative for AOA. Five hundred seventy infertile women enrolled for IVF-ET, AOA testing, corticosteroid therapy, and IVF-ET/ICSI. Around 40% of the patient who were AOA positive made antibodies to HSP90. Comparable clinical outcome and significance of AOA testing were established. AOA positive serum samples were sent periodically to reinvestigate the presence of AOA after corticosteroid therapy and women who turned AOA negative were taken up for IVF-ET. Of the 70/138 women in group 1 who were treated with corticosteroids and turned seronegative for AOA, 22/70 were poor responders and needed donor oocyte-recipient cycles. Results demonstrated that fertilization and clinical pregnancy rates between both groups were comparable. Nevertheless, it was also observed that there was a poor response to the stimulation protocol, smaller number of oocytes retrieved, and more spontaneous abortions in group 1 women. Hence, not all outcomes following the treatment were comparable between the two groups. Based on this data, we proposed that AOA could be used as a diagnostic marker for ovarian failure, and AOA testing could be included in the battery of tests investigating and treating infertility (Pires et al. 2011b).

7 HSP90 Interactome

Proteins rarely act alone and many times they team up and have intricate connections to undertake biological functions at both cellular and systems levels. A critical step towards unraveling the complex molecular relationships in living systems is the mapping of protein-to-protein physical "interactions." The complete map of protein interactions that can occur in a living organism is called the "*Interactome*" (Cusick et al. 2005). Using a complementary proteomics approach directed towards identification of novel proteins that interact with HSP90, an interactome for HSP90

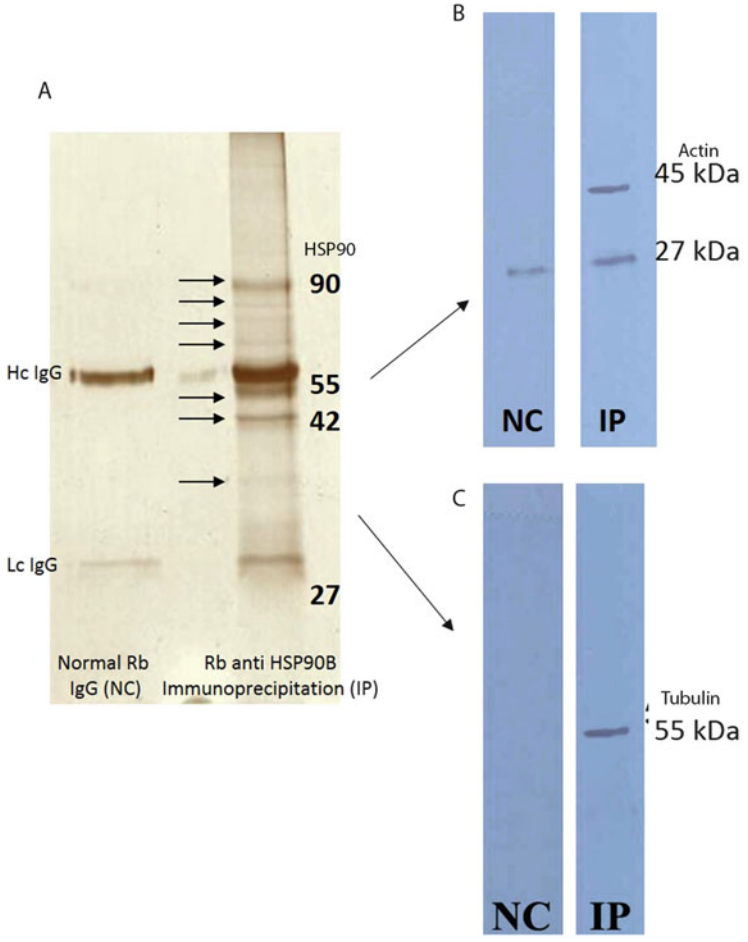

Fig. 2 Cytoskeletal proteins actin and tubulin co-immunoprecipitate with HSP90. Commercially available HSP90B antibody was used to pull down HSP90 protein as well as its binding partners as seen in the silver stained gel (**a**). A Western blot analysis was done on a parallel sample run and probed with actin (**b**) as well as tubulin (**c**) antibodies confirming that they were pulled down in a complex with HSP90

was established. These methods are co-immunoprecipitation, pull-downs with biotinylated geldanamycin, and immobilization of HSP90β on sepharose (Tsaytler et al. 2009). Among the proteins identified by this group, most of these were highly abundant proteins, including major HSP90 co-chaperones, structural proteins, ribosomal subunits, and metabolic and RNA-processing proteins. Also, novel HSP90 substrates at relatively low abundance were identified, such as the signaling proteins cell division protein kinase 3 (Cdk3) and tripartite motif containing 29 (TRIM29). Another study reported on the application of immunoprecipitation (IP) with endogenous HSP90, which yielded 39 interaction partners of HSP90 (Falsone et al. 2005). Of these reported proteins, only nine were previously established as HSP90 partners.

Identification of cytoskeletal proteins, ribosomal subunits, and metabolic and RNA-processing proteins strengthen the hypothesis that, besides the regulation of a specific set of proteins, HSP90 has a central function in several fundamental cellular processes (McClellan et al. 2007; Lotz et al. 2008). Thus, the IP and biotin-GA-mediated purification of structural proteins, including tubulin and kinesin, provides further evidence for the involvement of HSP90 in the assembly of the tubulin-based cytoskeleton network, cytokinesis, and cellular transport (McClellan et al. 2007; Te et al. 2007). Isolation of RNA-binding proteins and ribosomal subunits points to the suggested role of HSP90 in ribosomal subunit nuclear export and RNA processing and maintenance (Schlatter et al. 2002; Zhao et al. 2008). A pilot study done in the lab using a rabbit anti HSP90β as the IP antibody in a total crude ovarian extract (silver stain of the IP reaction shown in Fig. 2a), followed by Western blotting with actin and tubulin antibodies revealed IP of these 2 proteins at their appropriate known masses (Fig. 2b, c, respectively). This indicates that structural proteins such as actin and tubulin do interact with HSP90, and these interactions could be needed to maintain the cytoarchitecture of the cells.

8 Proposed Autoimmune-Mediated Ovarian Infertility via HSP90 as a Game Player

The HSPs play a critical role both in normal function and in the response to stress and are highly conserved in evolution, to an extent even greater than that of evidently essential proteins such as actin or myosin. HSP90 shows 60% amino acid identity with the corresponding yeast protein, 78% identity with the Drosophila protein, and a corresponding protein C62.5 has been identified in *E. coli* (Latchman and Isenberg 1994). Such evolutionary conservation of the HSPs thus results in homologues of the human HSPs being present in bacteria and other organisms such as parasitic protozoa which can infect humans. Such exogenous HSPs constitute the major target of the human immune response to these pathogens, and antibodies and

T cells against the appropriate exogenous HSPs have been detected in individuals infected with organisms as different as the mycobacteria and the protozoan parasites *Plasmodium falciparum* and *Schistosoma mansoni* (Biswas and Sharma 1994; Neumann et al. 1993). Although in these cases the antibodies appear to have a protective effect, in other cases the ability of antibodies and T cells directed against bacterial or protozoan HSPs to also react with the closely related endogenous human HSPs may result in autoimmunity leading to an autoimmune disease. It is more likely that, following the initial priming of the immune system by exposure to exogenous HSPs, some subsequent event involving the endogenous human HSPs is required to trigger the autoimmune response. Such an event could involve either the enhanced expression of the human HSPs or their expression on the cell surface, which can be brought about by a variety of stimuli such as microbial infection. Therefore, initial exposure to exogenous HSPs requires a second event, such as microbial infection, which results in upregulation of the human HSPs and/or their surface localization. This, in turn, induces antibodies and T cells primed against the bacterial or protozoan proteins reacting with the human proteins, leading to autoimmune disease.

In view of this, we propose that a majority of infertile women could have anti-HSP90β antibodies in circulation as a result of prolonged or repeated asymptomatic chronic infections early in life. In the course of a woman's reproductive life, these antibodies could then target the ovarian antigens (e.g., by exposure to the immune system due to accidents, trauma, or immune system memory), leading to early ovarian failure. This may be relevant to human reproduction because many couples with fertility problems have had a previously undetected genital tract infection (Witkin et al. 1994). The constitutive β form of HSP90 is known to be expressed at high levels during preimplantation mouse embryo development. Therefore, the presence of anti-HSP90β antibodies in women during early pregnancy is likely to have detrimental consequences. In parallel, monoclonal antibodies to mammalian heat shock proteins were also shown to impair mouse embryo development in vitro (Neuer et al. 1998).

The precise mechanism of anti-HSP90 antibody-related inhibition of embryo development and ovarian failure has not been reported. Recent reports have suggested that the penetration of autoantibodies into living cells participate in the pathogenesis of diverse autoimmune diseases. For instance, autoantibodies to HSP27 (also known as HSPB1), which are found in patients with glaucoma, have been shown to penetrate into human retinal neuronal cells and induce their active death, most likely by inactivating the ability of HSP27 to stabilize the actin cytoskeleton, suggesting a pathogenic role of these antibodies (Ruiz-Arguelles and Alarcon-Segovia 2001). Also, there is increasing evidence to suggest the presence of HSP90 on the cell surface (Calvert et al. 2003; Eustace and Jay 2004; Sidera et al. 2008) thereby making it accessible to the autoantibodies. Thus, the mere presence of these autoantibodies in the circulation may not only bring detrimental effects by binding to the surface HSP90, but they can also get internalized into the cell and destroy the ovarian cytoarchitecture.

So to reiterate, a persistent microbial infection in some of these women may mount a primary response to microbial HSP90 (Latchman and Isenberg 1994). Any accidental exposure could then account for a secondary immune response (by molecular mimicry). This could reactivate B lymphocytes previously sensitized to microbial HSP90 (by clonal expansion – immune system has memory). The humoral arm of the immune system would then get activated leading to increased production of anti-HSP90 antibodies. This probably could disturb immune regulating mechanisms necessary for oocyte and embryo development and maintenance.

At the cell structural level, it can be speculated that HSP90 associates itself with filamentous actin (Koyasu et al. 1986; Kellermayer and Csermely 1995). It is also well documented that HSP90 also binds to tubulin (Redmond et al. 1989; Czar et al. 1996). In stress conditions, HSP90 acts as a chaperone. During an environmental stress, which is exhibited by the presence of low ATP, this is known to be detrimental to these cytoskeletal frameworks (Loktionova et al. 1996) and if women have antibodies to HSP90 in their circulation, the possible interactions between HSP90 (in this case functioning as a molecular chaperone) and the cytoskeletal proteins may be disturbed and destroyed. Thus, there could likely be a collapse of the ovarian cytoarchitecture as the main role of HSP90 as a chaperone is to maintain this cytoskeletal framework. A proposed model leading to autoimmune infertility via HSP90 as a game player has been schematically depicted in Fig. 3.

9 Summary, Outlook, and Future Directions

HSP90 is a ubiquitous and an essential eukaryotic molecular chaperone that stabilizes a large set of client proteins, many of which are involved in various pathways. This chapter has elucidated that HSP90 plays an important and indispensable role in ovarian biology and pathology (Pires and Khole 2009a, b; Pires 2010; Pires et al. 2011a, b, 2013). The driving theme that is of interest to many is to be able to differentiate in its *housekeeping role*, in comparison to its *pathobiochemical role*. One of the important steps in the quest for HSP90 clients is to ensure its validity and to design experimental system/s to address more specific questions concerning mechanism and physiological importance related to a disease state. Because HSP90 also plays an important role in activation of the immune system, its pharmacological inhibition has increasingly become the focus of research on autoimmune diseases. There is a need to further characterize this chaperone and explore strategies for its utilization as a theranostic (therapeutics and diagnostics) agent.

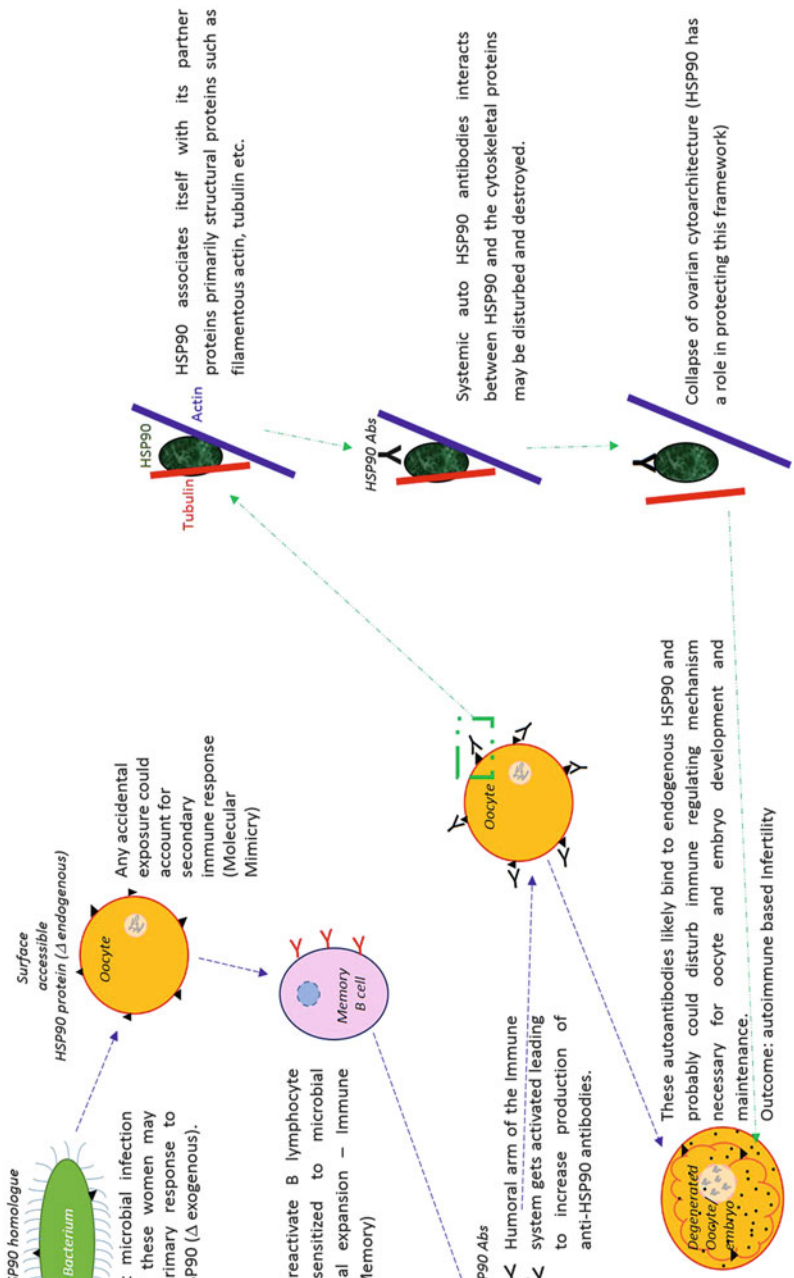

Fig. 3 A proposed model involving HSP90 as a key player in an outcome leading to autoimmune ovarian failure and infertility

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The Role of Heat Shock Factors in Mammalian Spermatogenesis

Wieslawa Widlak and Natalia Vydra

Abstract Heat shock transcription factors (HSFs), as regulators of heat shock proteins (HSPs) expression, are well known for their cytoprotective functions during cellular stress. They also play important yet less recognized roles in gametogenesis. All HSF family members are expressed during mammalian spermatogenesis, mainly in spermatocytes and round spermatids which are characterized by extensive chromatin remodeling. Different HSFs could cooperate to maintain proper spermatogenesis. Cooperation of HSF1 and HSF2 is especially well established since their double knockout results in meiosis arrest, spermatocyte apoptosis, and male infertility. Both factors are also involved in the repackaging of the DNA during spermatid differentiation. They can form heterotrimers regulating the basal level of transcription of target genes. Moreover, HSF1/HSF2 interactions are lost in elevated temperatures which can impair the transcription of genes essential for spermatogenesis. In most mammals, spermatogenesis occurs a few degrees below the body temperature and spermatogenic cells are extremely heat-sensitive. Pro-survival pathways are not induced by heat stress (e.g., cryptorchidism) in meiotic and postmeiotic cells. Instead, male germ cells are actively eliminated by apoptosis, which prevents transition of the potentially damaged genetic material to the next generation. Such a response depends on the transcriptional activity of HSF1 which in contrary to most somatic cells, acts as a proapoptotic factor in spermatogenic cells. HSF1 activation could be the main trigger of impaired spermatogenesis related not only to elevated temperature but also to other stress conditions; therefore, HSF1 has been proposed to be the quality control factor in male germ cells.

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1 Spermatogenesis Overview

Spermatogenesis is a tightly coordinated developmental process under hormonal control which enables the continuous production of sperm: from initial spermatogonial diploid cells, millions of haploid spermatozoa are produced daily that are able to fertilize a mature oocyte. In mammals, the process occurs in the seminiferous tubules of the adult male testes. Seminiferous tubules, the functional units of the mammalian testis, are composed of epithelium containing germinal cells and supportive Sertoli cells localized close to the tubule membrane. Sertoli cells, besides providing a nourishing function, maintain the integrity of spermatogenesis by forming the blood–testis barrier (rev. in: Brehm and Steger 2005). The interstitial, testosterone producing Leydig cells fill the extratubular space (rev. in: Davidoff et al. 2009). In the course of spermatogenesis, the germ cells continuously undergo mitotic, meiotic, and postmeiotic phases of maturation, moving towards the center of the seminiferous tubules. The following developmental stages are thereby passed through: spermatogonia (A, intermediate, and B), which divide

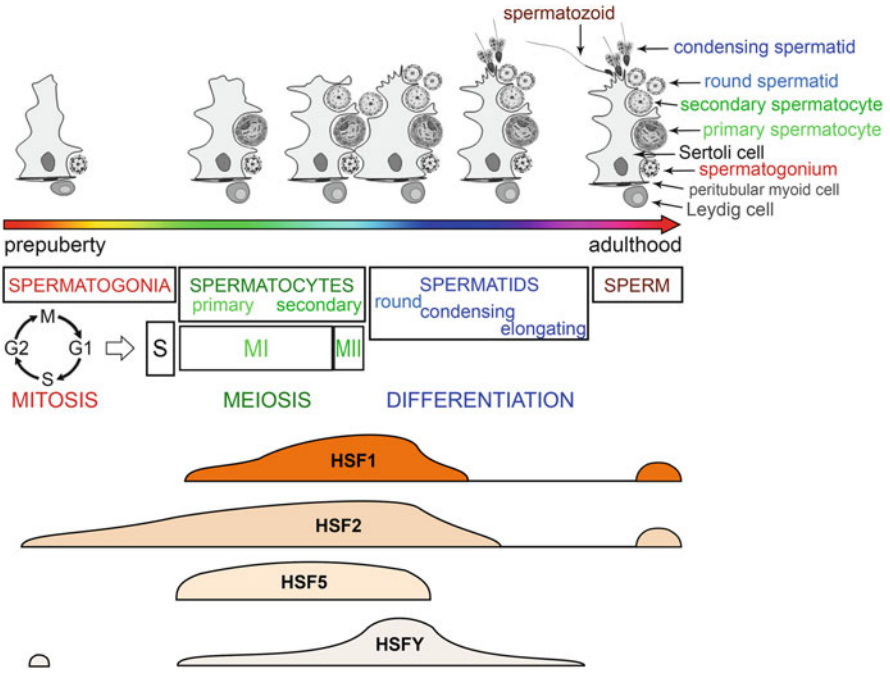

Fig. 1 A scheme of mammalian spermatogenesis and pattern of HSFs expression. The *upper schematic drawing* shows the cellular composition of developing testes (from prepuberty to adulthood). Developing germ cells are imbedded in a somatic Sertoli cell. HSFs are expressed predominantly in spermatocytes and spermatids undergoing meiosis and differentiation, respectively. Distribution of HSF3, HSF4, and HSFX in testes has not been determined yet. M, mitosis; G1, gap 1; S, synthesis of DNA; G2, gap 2; MI, meiosis I; MII, meiosis II

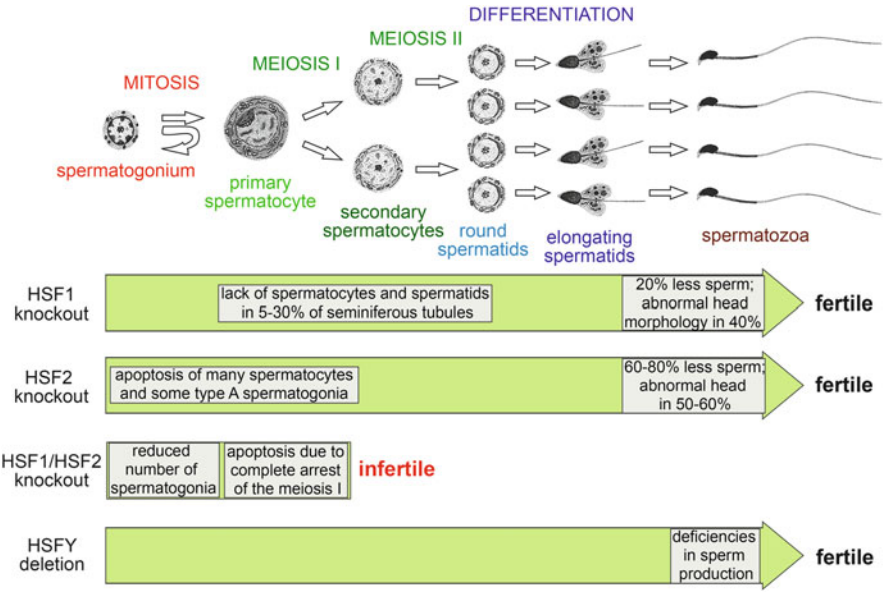

Fig. 2 Contribution of HSFs to male germ cell development. Consequences of HSFs loss are summarized based on mouse knockout models (HSF1 and HSF2) and naturally occurring microdeletions in the human genome (HSFY)

mitotically, spermatocytes (primary and secondary), which divide meiotically, and spermatids, which undergo morphological changes and differentiate into spermatozoa (Figs. 1 and 2). The duration of spermatogenesis varies considerably between species ranging from 35 to 70 days. During the prophase of the first meiotic division (which is the longest phase of meiosis), chromosomal synapsis and genetic recombination take place. After the completion of meiosis, during differentiation of spermatids, histones are replaced by transition proteins, then by protamines, which enables the tight packing of the paternal DNA, so it can be delivered unaffected to the oocyte (rev. in: Toshimori 2009). Spermatogenesis proceeds in synchronized waves along the seminiferous tubules, and every given cross section of the tubule contains only certain cell types in a specific combination (Kotaja et al. 2004). Maintenance of the proper ratio of each type of cell in the tubule is necessary for coordinated germ cell differentiation. Excessive (as well as aberrant) cells are removed by apoptosis, which is an important mechanism providing the homeostasis of male germ cells (rev. in: Shukla et al. 2012).

Spermatogenesis is temperature-dependent in most mammals. It occurs optimally at a temperature substantially lower (by around 4–5 °C) than the normal body temperature. Elevated testicular temperature has a detrimental effect on spermatogenesis and can compromise sperm quality and increase the risk of male infertility. A number of external and internal factors could disturb thermoregulation of the testes (which normally is controlled by the scrotum) and increase the risk of

abnormal spermatogenesis. These thermogenic factors include lifestyle and behavioral factors, occupational and environmental factors (external factors), and clinical factors resulting from pathophysiological conditions (internal factors). Impaired spermatogenesis related to elevated temperature is observed in certain disorders such as cryptorchidism, retractile testes, acute febrile diseases, obesity, varicocele, or as a result of occupational heat exposure (bakers, welders, drivers, or computer operators) and in men preferring a sitting lifestyle (rev. in: Agarwal et al. 2008; Skakkebaek et al. 2016).

The morphological changes that take place in seminiferous epithelium under an elevated scrotal temperature are well described using animal models. Reduced weight and smaller size of testes, disorganization of the germinal epithelium with concurrent reduced cell population, appearance of multi-giant cells, and reduced fertility were commonly observed in mouse and rat testes subjected to experimental cryptorchidism or hot baths (Reid et al. 1981; Loughlin et al. 1991; Yin et al. 1997; Lue et al. 1999; Rockett et al. 2001; Chaki et al. 2005). A significant decrease in sperm concentration and total sperm count were also observed in men exposed to transient scrotal hyperthermia (Rao et al. 2015). The most temperature-sensitive germ cells are primary spermatocytes (in the first meiotic division) (Yin et al. 1997; Rockett et al. 2001; Khan and Brown 2002). The loss of testicular germ cells triggered by heat shock occurs primarily by apoptosis. Damage of spermatocytes is manifested by giant cell formation, karyolysis, and appearance of abnormal chromosomal patterns as well. Moreover, decreased repopulation of seminiferous epithelium was observed in mice exposed to heat that could result from impairment of stem cells and delay in the reappearance of spermatocytes and spermatids (Gasinska and Hill 1990).

Exposition of most somatic cells to sublethal heat conditions induces a cytoprotective reaction called the heat shock response (rev. in: Richter et al. 2010). The fundamental component of this reaction is de novo synthesis of heat shock proteins (HSPs), which is regulated by heat shock transcription factors (HSFs). Accumulation of heat shock proteins renders somatic cells thermotolerant and allows them to survive subsequent otherwise lethal heat stress. However, diverse mechanisms are induced by elevated temperatures in spermatogenic cells, which are described below.

2 General Characteristics of Heat Shock Transcription Factors

Heat shock factors are well known as transcriptional regulators of genes encoding heat shock proteins, functioning as molecular chaperones. Eukaryotic cells express a number of different classes of HSPs, which can differ by their molecular weight, pattern of expression (constitutive vs. inducible), or cell localization (Kampinga et al. 2009). Expression of some HSPs is developmentally regulated or restricted to

specific cells (rev. in: Rupik et al. 2011; Ji et al. 2012; Dun et al. 2012). The general function of HSPs is to assist protein folding either during de novo synthesis or under stress conditions associated with protein misfolding. HSPs accomplish this by binding to stretches of hydrophobic amino acids in misfolded proteins and thus prevent formation of protein aggregates (rev. in: Vabulas et al. 2010). Besides the regulation of cell adaptation to stress mediated by induction of HSPs expression, members of the HSF family contribute to processes associated with development and growth. This function is probably associated with regulation of genes involved in diverse cellular processes that extend far beyond protein folding (which is the general role of HSPs).

The mammalian HSF family includes several members: HSF1 through HSF5, HSFY, and HSFX, which are grouped in one family due to high similarity of their DNA-binding domain. This domain enables binding to specific regions in the genome called Heat Shock Elements (HSEs). HSE consensus sequence is a tandem array of at least three oppositely oriented "nGAAn" motifs or a degenerate version thereof. In promoters of *HSP* genes, a number of such pentanucleotides in HSE can range from three to eight. Also the number of functional HSEs varies. The HSE architecture is an important determinant of specific HSF binding during gene regulation in diverse cellular processes (Yamamoto et al. 2009).

Although members of the HSF family have some common structural features, they are functionally distinct (for more information about the structure and properties of HSFs see, e.g., in: Fujimoto and Nakai 2010; Xu et al. 2012; Vydra et al. 2014). They generally exist in several isoforms and can undergo multiple post-translational modifications (phosphorylation, acetylation, sumoylation, ubiquitination). Moreover, they can interact with each other as well as with hundreds of different proteins (depending on the context) that modulate their activity. HSF1 (as well as avian HSF3, which among mammals was identified only in mice; Fujimoto et al. 2010) is activated by heat shock and numerous forms of physiological stress. HSF2 and HSF4 are considered as developmental factors, while the function of HSF5, HSFY, and HSFX, although not exactly known, is putatively associated with gametogenesis.

HSF1 is the main regulator of the heat shock response; thus, it has been studied the most extensively yet. Under physiological conditions, it exists as an inactive monomer in complexes with HSPs as well as other proteins. During stress conditions, when the level of unfolded proteins increases, HSPs are released from complexes with HSF1 and serve as molecular chaperones for unfolded molecules. An elevated level of unbound HSF1 promotes its trimerization, hyperphosphorylation, and high affinity binding to HSEs (each DNA-binding domain in the trimer recognizes a single nGAAn motif), which initiates the transcription of HSF1-dependent genes (rev. in: Pirkkala et al. 2001; Voellmy 2004; Fujimoto and Nakai 2010). HSF2 is supposed to form inactive dimers at physiological temperature and under several stress conditions trimerizes to a transcriptionally active form (Sistonen et al. 1994). No phosphorylation of HSF2 has been reported (although ubiquitination and sumoylation are possible) (Xu et al. 2012). HSF2 is a short-lived protein regulated mainly at the level of its expression. It can

autoregulate its own transcription (Park et al. 2015) and can be posttranscriptionally regulated by miR-18, which belongs to the Oncomir-1 cluster of microRNA (Björk et al. 2010). HSF3 also exists as a dimer at physiological conditions (Nakai and Ishikawa 2000), while HSF4 forms a transcriptionally competent trimer even at physiological temperature (Nakai et al. 1997).

3 The Role of HSFs in Spermatogenesis

3.1 Patterns of HSF Expression in Mammalian Testes

According to publicly available databases (e.g., Human Protein Atlas available from www.proteinatlas.org and BioGPS: http://biogps.org; Uhlén et al. 2015; Wu et al. 2016), all HSF family members are expressed in mammalian testes; however, HSF5 and HSFY are expressed exclusively in testes, while expression of the other family members is more ubiquitous. Among them, HSF2 is expressed primarily in testes, then in brain, whereas HSF4 is primarily in lenses.

In mouse and rat testes, HSF1 protein was detected in spermatocytes (mainly in nuclei, although it is excluded from metaphase plate during meiotic divisions) and round spermatids, then its level diminishes in elongating spermatids (Alastalo et al. 1998; Akerfelt et al. 2010). A very similar pattern of expression was observed in the case of HSF2 (Sarge et al. 1994; Goodson et al. 1995; Alastalo et al. 1998), but it is possible that *Hsf2* gene expression starts in spermatogonia and is retained up to elongating spermatids (Kallio et al. 2002; Björk et al. 2010; Chalmel et al. 2012). In rat testes, HSF2 was additionally found in cytoplasmic regions identified as cytoplasmic bridges between germ cells (Alastalo et al. 1998). Furthermore, both HSF1 and HSF2 were found in the heads of epididymal spermatozoa (Wilkerson et al. 2008). The testes express predominantly the larger α isoform of HSF1 and HSF2 (Goodson et al. 1995; Neueder et al. 2014). It is noteworthy that transcription of HSF1 is downregulated by the androgen receptor in Sertoli cells; thus, it is under the influence of testosterone (Yang et al. 2014).

Expression of HSF5 protein is restricted to spermatocytes and round spermatids (documented in human and rat testes; Chalmel et al. 2012); thus, it is essentially the same as HSF1 and HSF2. Also human HSFY was found in these stages of spermatogenesis and additionally in elongated spermatids or occasionally in type A spermatogonia and Sertoli cells (Shinka et al. 2004; Sato et al. 2006). In situ hybridization revealed that mouse *Hsfy* was predominantly expressed in round spermatids (Kinoshita et al. 2006). Up to date, there is no information on localization of HSF3, HSF4, and HSFX proteins in spermatogenic cells. Summarizing, almost all HSFs are expressed at the highest level in spermatocytes and round spermatids (Fig. 1), which coincides with the phases of active transcription, synaptonemal complex formation, crossing-over, and intensive chromatin remodeling that take place in these cells.

3.2 Relevance of HSFs for Normal Spermatogenesis

To understand the physiological roles of HSFs in vivo, knockout mice for these factors were generated (rev. in: Christians and Benjamin 2006). Targeting of the *Hsf1* gene resulted in increased prenatal lethality (as a result of defects in the placenta) and postnatal growth retardation. Although many physiological functions were affected (e.g., thermotolerance, systemic body temperature regulation, redox homeostasis and antioxidative defenses, immune response, motor activity, smell, hearing, memory, and others), under normal conditions such mice could survive to late adulthood. However, *Hsf1* knockout females were infertile due to defects in oogenesis and preimplantation development, while males exhibited normal reproductive ability yet produced about 20% less sperm than wild-type mice (Xiao et al. 1999; Salmand et al. 2008; rev. in: Abane and Mezger 2010). Moreover, approximately 40% of epididymal spermatozoa displayed abnormal head morphology. Reduced number of sperm was a consequence of a slightly disrupted spermatogenesis: disorganized or missing layers of germ cells were found in 5–30% of the *Hsf1*−/− seminiferous tubules. Most of the affected tubules contained only spermatogonia, some of them additionally contained spermatocytes, but all disrupted tubules lacked spermatids. Abnormal sperm head morphology of *Hsf1*−/− sperm could arise from defects in the chromatin packing due to disturbed replacement of transition proteins with protamines (Fig. 2) (Salmand et al. 2008; Akerfelt et al. 2010).

Defective spermatogenesis was also observed in *Hsf2* null mice, whereas other defects included increased embryonic lethality, nervous system developmental disturbances, female hypofertility, and ovary abnormalities (rev. in: Abane and Mezger 2010). The synaptonemal complex, which is formed between homologous chromosomes during the pachytene stage of meiotic division, was frequently disorganized in such mice (Kallio et al. 2002). Many primary spermatocytes and some type A spermatogonia died by apoptosis. As a result, testes of *Hsf2*−/− males were significantly smaller and the number of epididymal sperms was reduced by 60–80% in comparison to wild-type animals (Kallio et al. 2002; Wang et al. 2003). Moreover, similarly to *Hsf1* knockout mice, sperm head abnormalities were detected in more than half of spermatozoa that suggested defects in the chromatin condensation (Akerfelt et al. 2008). Despite markedly reduced sperm count and quality, only a small reduction in fertility was observed in *Hsf2*−/− male mice (Fig. 2). More severe defects of spermatogenesis (block at the spermatocyte stage and infertility) were detected in men with a dominant-negative mutant form of HSF2 (Mou et al. 2013). However, such an inhibitory mutation was detected only in one of 766 patients infertile due to unknown causes, and coexisting alterations in other important genes cannot be excluded.

Importantly, mice with the double knockout of both *Hsf1* and *Hsf2* exhibited severe defects of spermatogenesis associated with a reduced number of dividing spermatogonia and arrest of spermatogenesis at pachytene stage of the first meiotic division leading to apoptosis. As a consequence, such males completely lacked

postmeiotic cells and were infertile, despite normal mating performance (Fig. 2). This observation indicates the complementary roles of HSF1 and HSF2 in spermatogenesis (Wang et al. 2004).

Knockout models reveal the lens-specific role of HSF4 because *Hsf4*-null mice developed cataract. However, males and females were fertile, and the histology of the testis and ovary was normal (Fujimoto et al. 2004). Other HSF (i.e., HSF3, HSF5, HSFY, and HSFX) mouse knockout models have not been established yet. However, a few cases of *HSFY* deletions were found in human. Functional *HSFY* and *HSFX* genes both exist in two identical copies on the human Y or X chromosome, respectively (Bhowmick et al. 2006). *HSFY1* and *HSFY2* are located within rapidly evolving male-specific region of Y (MSY) that contains several testis-specific genes organized in palindromes. Deletions in MSY are associated with deteriorated spermatogenesis and azoospermia (complete absence of spermatozoa in the ejaculate). *HSFY* genes are usually deleted together with other protein coding genes from that region. Microdeletions restricted to *HSFY* locus could also occur. They result in deficiencies of the sperm production; however, spermatogenesis is not completely arrested (Fig. 2). Thus, it was concluded that *HSFYs* (and overlapping noncoding genes) are not absolutely required for human male fertility. On the other hand, functional *HSFY* genes have been conserved on the Y chromosome for 100,000 years during the evolution of modern humans, indicating that HSFY does make a real contribution to the reproductive fitness of men (Kichine et al. 2012). Nevertheless, detection of *HSFY* deletion and assessment of *HSFY* mRNA level were proposed as useful diagnostic markers for the presence of retrievable testicular sperm in assisted reproductive technologies (Stahl et al. 2011).

In conclusion, a largely overlapping distribution of HSFs in spermatogenic cells and insights from knockout models implicate that different HSFs could cooperate to maintain proper spermatogenesis. Moreover, it cannot be excluded that HSFs may be able to some extent compensate each other.

3.3 Interplay between HSF1 and HSF2 in Spermatogenesis

Although HSF1 and HSF2 are functionally distinct, they can cooperate either under physiological conditions or during stress (Mathew et al. 2001; He et al. 2003; Ostling et al. 2007; Shinkawa et al. 2011). Co-localization of HSF1 and HSF2, most probably in the form of heterotrimers, was shown in the nuclear stress granules/bodies, which were formed in response to heat shock in human cells (Alastalo et al. 2003). Moreover, HSF1-dependent transcription could be modulated by the HSF1/HSF2 ratio (He et al. 2003; Loison et al. 2006; Ostling et al. 2007; Sandqvist et al. 2009; Elsing et al. 2014). HSF1/HSF2 complexes/heterotrimers also exist in mouse spermatogenic cells (Sandqvist et al. 2009; Akerfelt et al. 2010; Korfanty et al. 2014). Complete male infertility observed specifically in double knockout mice due to arrest of meiosis (Wang et al. 2004) suggests that HSF1/HSF2 cooperation could be critical for proper spermatogenesis.

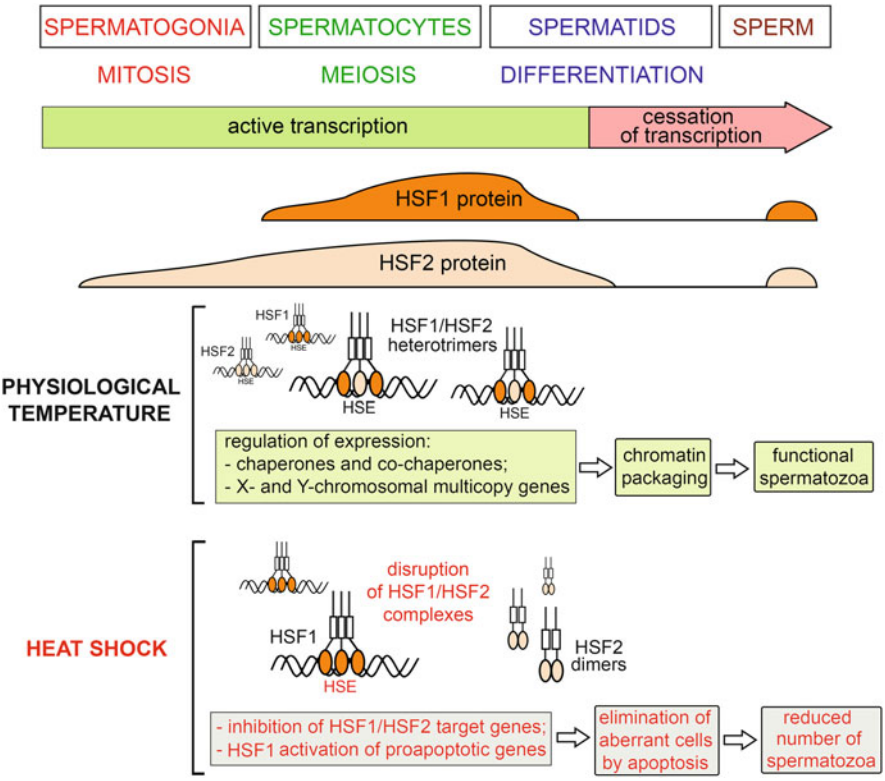

Fig. 3 Interplay between HSF1 and HSF2 in spermatogenesis at physiological and elevated temperatures. Observed cooperation of HSF1 and HSF2 in spermatocytes and round spermatids is critical for normal spermatogenesis. Heat shock-induced disruption of HSF1/HSF2 interactions is connected with release of HSF2 from chromatin and increased HSF1 binding. Proapoptotic genes could be activated by HSF1 leading to the elimination of damaged germ cells. HSE, heat shock element

HSE-binding activity of both HSF1 and HSF2 was detected in mouse testes (Fiorenza et al. 1995). However, genome-wide analyses of HSF1 and HSF2 DNA target sites revealed that both transcription factors generally occupy different genomic targets, and only ~15% binding sites are shared (Akerfelt et al. 2008, 2010). Genes whose promoters are co-occupied in mouse spermatogenic cells by both HSF1 and HSF2 encode primarily for chaperones and co-chaperones that facilitate protein folding (Korfanty et al. 2014). Thus, both factors could participate in the regulation of the basal level of transcription of these genes at the physiological temperature (Fig. 3). Interestingly, expression of the testis-specific *Hspa2* gene, which is restricted to spermatocytes and spermatids and is indispensable for spermatogenesis (rev. in: Scieglinska and Krawczyk 2015), seems to be independent on HSF1 or HSF2 binding (Wang et al. 2004; Korfanty et al. 2014).

It has to be noted that there is a huge inconsistency among HSFs binding sites mapped at the physiological temperature by different technologies (Akerfelt et al. 2008, 2010; Kus-Liśkiewicz et al. 2013; Korfanty et al. 2014), which suggests that the binding is relatively weak in such conditions. Observed variability could result from a transient sequence-independent chromatin binding, corresponding to the HSFs search for more specific targets that was suggested for HSF1 by Herbomel et al. (2013).

The exact HSF1 and HSF2 role in specific germ cell types is unknown; however, their involvement in the regulation of postmeiotic expression of certain X- and Y-chromosomal multicopy genes (e.g., *Sly*, *Slx*, *Ssty1/2*) required for the correct packing of the chromatin in sperm was documented (Fig. 3) (Akerfelt et al. 2008; Akerfelt et al. 2010). The X and Y chromosomes are largely non-homologous; hence, they synapse during meiosis in their short homologous regions only, while unsynapsed regions are silenced and incorporated into a distinct chromatin domain at the cellular periphery called the sex body (rev. in: Turner 2007). HSF1 and HSF2 binding allows the multicopy genes to escape postmeiotic sex chromosome repression; hence these genes can be active in round spermatids. Consistently, lack of HSF1 or HSF2 results in deregulated expression of the multicopy genes and altered sperm head morphology equivalent to that observed in mutant males with partial deletion of the MSY long arm (Ellis et al. 2005).

HSF2 occupies hundreds of loci in the chromatin during mitosis; among others it binds to the promoter of inducible *HSP70* (*HSPA1*) gene preventing its full compaction (Xing et al. 2005). Such gene bookmarking maintains the promoter in a transcription competent state, and its induction could occur even in the early G1 phase if stress were to arise. Similar mechanism of *Hsp70* bookmarking in condensed chromatin was demonstrated in mouse spermatogenic cells. HSF2 linked to the promoter of *Hspa1b* gene already in late spermatids most likely enables subsequent binding of HSF1 as well as SP1 transcription factor in mature sperm. Keeping the promoter chromatin in open conformation would make the *Hspa1b* gene ready for early activation that occurs shortly after fertilization in the one-cell stage embryo (Christians et al. 1995; Wilkerson et al. 2008). In contrary to HSF2, HSF1 is rapidly excluded from chromatin when the metaphase plate is formed, both in mitosis and meiosis (Vihervaara et al. 2013).

3.4 Contribution of HSF1 and HSF2 to the Heat Shock Response in Testes

Extensive studies revealed that HSF1 activation protects cells by inducing a set of HSPs, which enable cells to survive otherwise lethal stress. This function of HSF1 has been described in various model systems ranging from cell culture to stress-response analyses at the organismal level, such as yeast, nematodes, and rodents. Accordingly, accelerated heat-induced apoptosis was commonly observed in cells

with reduced HSPs expression due to the HSF1 downregulation (McMillan et al. 1998; Xiao et al. 1999). Searching for a temperature set point for induction of HSF1 binding to DNA revealed that HSF1 activation takes place at temperatures of 41 °C or higher in most mammalian cell types and tissues (liver, kidney, lung), whereas in testes HSF1 is activated at significantly lower temperatures (35–38 °C) (Sarge 1995). This phenomenon is a unique property of male germ cells, with a minor exception of T-lymphocytes where HSF1 is activated at febrile temperatures (i.e., 39 °C and above; Gothard et al. 2003). Somatic types of testicular cells (peritubular myoid and Leydig cells) exhibit HSF1 activation profile identical to that observed in other somatic mammalian cells (Sarge 1995). It has to be emphasized that despite HSF1 activation, pro-survival pathways are not induced in meiotic and postmeiotic cells in response to stress. Transcription of major heat shock genes is induced only in somatic compartment of testes and possibly in premeiotic germ cells, although activated HSF1 binds to *Hsp* gene promoters also in spermatocytes (Huang et al. 2001; Izu et al. 2004; Kus-Liśkiewicz et al. 2013). Moreover, the constitutively expressed testis-specific variants of *Hsp70* (*Hspa2* and *Hspa1l*) genes are downregulated following heat shock (Widlak et al. 2007a). Furthermore, ectopic expression of HSPA1 (the major heat inducible HSP70 family member) do not protect spermatogenic cells from apoptosis induced by heat shock (Widlak et al. 2007b).

Severity and consequences of heat shock depend on temperature and duration of exposure (rev. in: Balogh et al. 2013). Hence, responses of spermatogenic cells to hyperthermia at 37–38 °C (e.g., in cryptorchidism) and at 42–43 °C are different. Even relatively short (15–20 min) heat shock at 43 °C resulted rather in changes of critical proteins' localization then in changes of their levels. Redistribution of BAX from cytoplasmic to perinuclear space, release of cytochrome c, and subsequent activation of the initiator caspase 9 and the executioner caspases 3, 6, and 7, as well as resulting chromatin fragmentation and PARP cleavage were observed within 0.5–2 h after heating (Yamamoto et al. 2000; Hikim et al. 2003). In experimental cryptorchidism (i.e., approximately at 37 °C), massive apoptosis is observed much later, in three to four day time frame (Barqawi et al. 2004; Chaki et al. 2005; Tao et al. 2006). Differences in the early response of spermatogenic cells to the temperature shift up to 38 °C (mild stress) or 43 °C (acute stress) were also visible at the transcriptional level. At the acute stress, only ~2.5% of all transcribed genes were affected (mostly repressed), whereas about 10% of genes were affected at the mild stress (Kus-Liśkiewicz et al. 2013). The rapid early response to the severe stress observed in spermatogenic cells seems to be independent of activation of HSF1 as the transcription factor. However, significantly lower number of spermatocytes undergoing apoptosis induced both by mild or severe heat shock in HSF1 deficient mice suggests some role of HSF1 also in this early response (Izu et al. 2004).

HSF2 protein is less stable than HSF1. It is ubiquitylated by the ubiquitin E3 ligase anaphase-promoting complex/cyclosome (APC/C) and directed to proteasomal degradation within one hour in response to stress (Ahlskog et al. 2010). Thus, the general ability of HSF2 to bind HSEs diminishes along with

heat shock duration and temperature increase (Sarge et al. 1991). However, following heat shock both HSF1 and HSF2 could bind concurrently in some HSPs promoters (Trinklein et al. 2004; Ostling et al. 2007; Ahlskog et al. 2010; Shinkawa et al. 2011). A ChIP-Seq study performed in human K562 erythroleukemia cells also showed the involvement of both HSFs in the regulation of genes coding for chaperones and co-chaperones, where the total number of HSF1 and HSF2 target loci was increased after temperature elevation (Vihervaara et al. 2013). A similar study performed in isolated mouse spermatogenic cells revealed that temperature elevation resulted in increased binding of HSF1 (mainly to promoters of chaperones and other stress-related genes), while HSF2 binding gradually decreased up to complete loss at 43 °C. In agreement with this observation, the number of HSF1/HSF2 complexes detected in spermatogenic cells in situ was markedly reduced at elevated temperatures (Korfanty et al. 2014). It is noteworthy that increased HSF1 binding was not associated with activation of the target genes transcription but rather with their repression upon hyperthermia (Kus-Liśkiewicz et al. 2013). This could be caused by disruption of HSF1–HSF2 interactions following the release of HSF2 from chromatin, which apparently resulted in the suppression of several genes essential for spermatogenesis during stress conditions (Fig. 3).

3.5 HSF1 as a Quality Control Factor in Male Germ Cells

Disturbed interactions of HSF1 and HSF2 and resulting misregulation of transcription of target genes could be an important consequence of temperature elevation in spermatogenic cells. However, it is also known that activation of HSF1 alone is sufficient to trigger apoptosis in meiotic and postmeiotic cells. Expression of mutated, constitutively active HSF1 in spermatogenic cells of transgenic mice leads to massive degeneration of seminiferous epithelium and male infertility (Nakai et al. 2000; Widłak et al. 2003). In contrary to the majority of somatic cells, where activation of HSF1 is an important element of the cytoprotective system, HSF1 acts as a proapoptotic factor in spermatocytes and spermatids (Fig. 4). Active HSF1 do not induce *Hsps* genes in spermatogenic cells, instead, it initiates caspase-3-dependent apoptosis to eliminate target cells (Vydra et al. 2006). Such HSF1-dependent mechanism apparently prevents transition of the potentially impaired genetic material to the next generation. Since huge amount of spermatozoa are produced daily, elimination of spermatogenic cells damaged during meiosis or differentiation seems to be a more reasonable strategy than their repair. Thus, HSF1 has been proposed to be a quality control factor in male germ cells (Izu et al. 2004).

Mechanisms of proapoptotic action of HSF1 are still not fully resolved. PHLDA1, whose expression is activated directly by HSF1 in some cells including mouse testes, could play an important role here. Overexpression of PHLDA1 may induce apoptosis depending on the cell context. As long as HSF1 (alone or in cooperation with HSF2) activates expression of *Hsps* genes, HSPs interact with

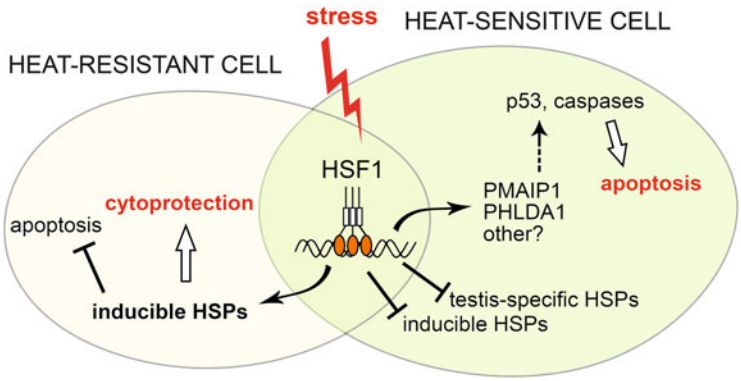

Fig. 4 Proposed model of the HSF1-dependent heat shock response in heat-resistant and heat-sensitive cells. HSF1 activated by different forms of cellular stress induces expression of heat shock proteins (HSPs) that prevent apoptosis and provide cytoprotection in heat-resistant cells (most somatic cells). Expression of HSPs (both inducible and testis-specific variants) is blocked in heat-sensitive meiotic and postmeiotic male germ cells. Instead, HSF1 activates expression of proapoptotic proteins (PMAIP1, PHLDA1) that in the absence of HSPs could induce apoptosis, which actively eliminates potentially impaired cells

PHLDA1 and inhibit cell death mediated by this protein. Since expression of *Hsps* is repressed in spermatogenic cells exposed to high temperatures, PHLDA1 could promote apoptosis when it does not form a complex with HSPs. In fact, heat-induced apoptosis is to some extent diminished in PHLDA1 null mice testes (Hayashida et al. 2006). HSF1 can also directly upregulate the expression of a strictly proapoptotic gene—*Pmaip1* (*Noxa*) after heat shock treatment (Korfanty et al. 2015). PMAIP1 is a proapoptotic member of the BCL2 family (rev. in: Ploner et al. 2008). Similarly to PHLDA1, overexpression of PMAIP1 can induce apoptosis (Oda et al. 2000). PMAIP1 promotes apoptosis by targeting the pro-survival protein MCL1 for proteasomal degradation. Involvement of PMAIP1 in heat-induced apoptosis was already shown in human acute lymphoblastic T-cells (Stankiewicz et al. 2009). Overexpression of HSP70 stabilized MCL1 protein levels in heat-shocked cells and prevented heat-induced BAX activation and apoptosis. It is noteworthy that *Pmaip1* is the most induced gene by active HSF1 in 15-day-old transgenic mice testes (when the transgene is already expressed in the first pachytene spermatocytes, while massive apoptosis is not visible yet; Vydra et al. 2006; http://dx.doi.org/10.6070/H4P26W5M). Interestingly, HSF1 binding was detected not in the promoter, but in the second intron of the *Pmaip1* gene (Korfanty et al. 2014).

Data presented above strongly suggests that cell fate on proteotoxic condition is determined by the balance between antiapoptotic HSPs and proapoptotic proteins including PHLDA1 and PMAIP1, which are differently regulated by HSF1 in heat-sensitive and heat-resistant cells. This paradoxical activation of both pro- and antiapoptotic mechanisms by HSF1 in response to stress ensures that neither aberrant cellular survival nor inappropriate cell death arises.

4 Summary and Future Directions

Decreased semen quality and sperm concentration are the major factors of male infertility observed over the past several decades in many areas of the world. The etiology of these declines is complex and not well understood, yet involvement of environmental stress is generally appreciated. Hence, explanation of the role of heat shock transcription factors and their cooperation during normal spermatogenesis as well as during cellular stress would apparently contribute to understanding the problem of male subfertility. Below, we have pointed out the relevant data which was addressed in the current review.

1. All HSF family members are expressed in mammalian testes, mostly in spermatocytes and round spermatids: HSF5 and HSFY are expressed exclusively in testes while expression of the other family members is more ubiquitous (although HSF2 is expressed primarily in testes).
2. HSF1, HSF2, or HSFY deficient males reveal some defects in spermatogenesis. Moreover, double HSF1 and HSF2 knockout is connected with complete arrest of meiosis and males infertility. Hence, cooperation of different HSFs in a proper maintenance of spermatogenesis is suggested.
3. HSF1 and HSF2 partially share genomic-binding sites, and in mouse spermatogenic cells both factors could cooperate in the regulation of the basal level of transcription of several genes coding for chaperones and co-chaperones that facilitate protein folding.
4. The postmeiotic expression of certain X- and Y-chromosomal multicopy genes (e.g., *Sly*, *Slx*, *Ssty1/2*) required for the correct packing of the chromatin in sperm depends on both HSF1 and HSF2; hence, a deficiency of HSF1 or HSF2 results in sperm head abnormalities.
5. HSF2 is not excluded from the condensing chromatin during meiosis maintaining some promoters in a transcription competent state; hence, binding of other transcription factors (including HSF1) and rapid induction of target genes remains possible.
6. Spermatogenesis requires a temperature substantially lower than normal body temperature; thus, an elevated testicular temperature has a detrimental effect on spermatogenesis and can compromise sperm quality and increase the risk of male infertility (primary spermatocytes during the first meiotic division are the most heat-sensitive).
7. Death of germ cells could be induced by either mild (37 °C) yet extended elevation of temperature exemplified by cryptorchidism (massive apoptosis observed after three-four days) or acute heat shock at 42–43 °C (massive apoptosis observed after a few hours).
8. Interactions between HSF1 and HSF2, which are crucial for correct spermatogenesis, are disrupted during heat shock, when activation of HSF1 and its increased binding to chromatin correlates with destabilization and decreased binding of HSF2; consequently, transcription of many genes essential for spermatogenesis is inhibited during stress.

9. HSF1, the main regulator of the heat shock response and important element of the cytoprotective system, is activated in spermatogenic cells at significantly lower temperatures than in somatic cells (starting from 35 to 41 °C, respectively).
10. Despite the HSF1 activation, pro-survival pathways are not induced in male meiotic and postmeiotic cells in response to stress: although activated HSF1 binds to promoters of *Hsp* genes in both spermatocytes and somatic cells, transcription of cytoprotective HSPs is induced only in the somatic compartment of testes. Moreover, the constitutively expressed testis-specific variants of *Hsp70* (*Hspa2* and *Hspa1l*) genes are downregulated following heat shock, and ectopic expression of HSPA1 (the major heat inducible HSP70 family member) do not protect spermatogenic cells from heat shock-induced apoptosis.
11. Transcriptionally active HSF1 induces caspase-3-dependent apoptosis in spermatocytes and spermatids, which allows the elimination of potentially damaged spermatogenic cells. Hence, HSF1 has been proposed to be a quality control factor that prevents transition of the potentially damaged genetic material to the next generation.
12. HSF1 may play a dual role in response to heat shock, either cytoprotective or cytotoxic. Besides the upregulation of cytoprotective HSPs, HSF1 can directly activate the expression of proapoptotic PHLDA1 and PMAIP1 (NOXA); thus, the final response to stress could be determined by the balance between antiapoptotic and proapoptotic factors regulated by HSF1 differentially in heat-sensitive and heat-resistant cells.
13. Molecular mechanisms of the proapoptotic action of HSF1 in heat-sensitive cells as well as the functions of other HSF family members (especially those testis-specific, i.e., HSF5 and HSFY) during spermatogenesis are not fully resolved and further studies are needed.

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Heat Shock Protein A2 (HSPA2): Regulatory Roles in Germ Cell Development and Sperm Function

Brett Nixon, Elizabeth G. Bromfield, Jinwei Cui, and Geoffry N. De Iuliis

Abstract Among the numerous families of heat shock protein (HSP) that have been implicated in the regulation of reproductive system development and function, those belonging to the 70 kDa HSP family have emerged as being indispensable for male fertility. In particular, the testis-enriched heat shock 70 kDa protein 2 (HSPA2) has been shown to be critical for the progression of germ cell differentiation during spermatogenesis in the mouse model. Beyond this developmentally important window, mounting evidence has also implicated HSPA2 in the functional transformation of the human sperm cell during their ascent of the female reproductive tract. Specifically, HSPA2 appears to coordinate the remodelling of specialised sperm domains overlying the anterior region of the sperm head compatible with their principle role in oocyte recognition. The fact that levels of the HSPA2 protein in mature spermatozoa tightly correlate with the efficacy of oocyte binding highlight its utility as a powerful prognostic biomarker of male fertility. In this chapter, we consider the unique structural and biochemical characteristics of HSPA2 that enable this heat shock protein to fulfil its prominent roles in orchestrating the morphological differentiation of male germ cells during spermatogenesis as well as their functional transformation during post-testicular sperm maturation.

1 Introduction

The overwhelming complexity of reproductive system development and function and the importance these processes hold in securing the reproductive fitness of an individual necessitate rigorous mechanisms of quality assurance. This is especially true in the case of spermatogenesis, an intricate process of cellular differentiation that culminates in the production of extraordinary numbers of spermatozoa, arguably one of the most highly specialised cells in biology (Hermo et al. 2010). The responsibility for ensuring the fidelity of this process falls, at least in part, to a suite

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of heat shock proteins (HSP), which act as folding catalysts with prominent roles in facilitating protein and peptide transport, stabilisation of nascent proteins, inhibition of protein aggregate formation, and the mediation of protein complex formation (Dun et al. 2012). The importance of heat shock proteins for sperm development also extends to synergistic roles associated with the functional transformation of these cells that occurs during their successive phases of post-testicular maturation within the male (epididymal maturation) and female reproductive tracts (capacitation) (Dun et al. 2012). This is particularly the case given that the functional remodelling of the sperm cells occurs in the complete absence of transcriptional and translational intervention.

Accordingly, compelling evidence now exists linking the dysregulation of HSP expression and/or function as causative agents in defective sperm function. In many cases of male sub-fertility, associated with not only our own species but also that of select animal models, targeted disruption of HSP proteins has the ability to arrest spermatogenesis, compromise sperm maturation, and inhibit fertilisation (Purandhar et al. 2014; Held et al. 2011; Audouard and Christians 2011; Adly et al. 2008; Held et al. 2006; Cayli et al. 2003; Feng et al. 2001; Timakov and Zhang 2001; Dix et al. 1996a; Boulanger et al. 1995). These proteins therefore hold considerable promise as targets for novel contraceptive strategies and as diagnostic biomarkers for male infertility. While many families of HSPs have been implicated in these combined processes, this chapter focuses on the heat shock 70 kDa protein 2 (HSPA2), a testis-enriched member of the 70 kDa heat shock protein (HSP70) family that promotes the folding, transport, and assembly of protein complexes and has been positively correlated with in vitro fertilisation success. In particular, we consider the structural and biochemical characteristics of HSPA2 in an attempt to shed light on the prominent roles assigned to this heat shock protein in terms of orchestrating the morphological differentiation of male germ cells during spermatogenesis as well as their functional transformation during sperm maturation.

2 Structural and Biochemical Characteristics of the Hsp70 Family

2.1 Overview of the Heat Shock Protein 70 Family

Heat shock proteins derive their name from their capacity to buffer cells against the potentially detrimental impact of exposure to a variety of environmental insults, including thermal stress (Ritossa 1996). As a reflection of their integral role within the protein homeostasis (proteostasis) network, HSPs are ubiquitously expressed across most cell types and within virtually all organisms across the phylogenetic tree (Ellis 1990). In this capacity, HSPs are responsible for facilitating the biogenesis, stabilisation, folding, trafficking, and degradation of proteins within the cell.

Such broad ranging functions are mirrored by a similar diversity of HSP families, which include: HSPH (HSP110), HSPC (HSP90), HSPA (HSP70), HSPD (HSP60), DNAJ (HSP40), and HSPB (small HSP) (Kampinga et al. 2009). These families are so assigned on the basis of their molecular weight and structural characteristics (Burel et al. 1992). However, additional defining features of the HSP families include their overlapping expression profiles and the interconnected nature of their functions (Bukau et al. 2006). Indeed, mounting evidence indicates that a complex interplay occurs between the different families of HSP's to underpin their archetypical protective roles, with dysregulation of such quality control pathways leading to a loss of proteostasis during ageing and disease (Yerbury et al. 2016).

In keeping with the intricate interplay of HSPs to deliver a cellular outcome, the core structural elements of most classes display a considerable degree of conservation. Such findings, however, raise intriguing questions regarding how the HSPs express their specificity of action. What we do understand is that specific pairing between families of HSPs form the basis of the regulatory mechanisms, and given the potential multitude of combinations between HSPs, this also lays a platform that could account for a broad diversity of function. Illustrative of such interactions, members of the HSP40 family are known to fulfil a key regulatory role in terms of modulating HSP70 activity, directing substrates to the protein and focusing this family to discrete subcellular compartments (Kampinga and Craig 2010). In addition, the HSP40–HSP70 partnership can subsequently engage HSP90s, which accept the substrate to assist in further folding or protein degradation pathways. While potentially over-simplistic, this description illustrates the importance of understanding not only the function of individual HSPs but also the network of HSP client proteins and other regulatory molecules that interact within the proteostasis pathways.

As the focus for this chapter, the 70 kDa HSPs (HSP70) rank among the most highly abundant and evolutionarily conserved members of the HSP family, with at least 13 gene products represented in the human proteome (Kampinga et al. 2009; Radons 2016). Members of the HSP70 family were originally identified among those proteins intimately associated with enhancing cell survival and recovery following exposure to various external stress interventions. As early as 1984, it was suggested that the ability of HSP70 to enhance the recovery of stressed cells was mediated by its capacity to catalyse the reassembly of damaged ribosomal proteins (Pelham 1984). Subsequent research revealed that this chaperoning function was a common feature among HSP70 proteins and that it was essential for not only protection against stress but also for the house-keeping roles assigned to constitutively expressed HSP70 proteins in non-stressed cells [reviewed in Kampinga et al. (2009) and Radons (2016)]. These functions now extend to the transport of proteins between cellular compartments, degradation of unstable and misfolded proteins, prevention and dissolution of protein aggregates, folding and refolding of proteins, uncoating of clathrin coated vesicles, and control of regulatory proteins. To clarify how HSP70s regulate such diverse functions, we next give consideration to the structural features of the HSP70 family, with particular

reference to how they orchestrate productive interactions with diverse client proteins within the cellular proteome.

2.2 *Structural Features of the Heat Shock Protein 70 Family*

It is understood that classes of newly translated proteins can fold independently of HSPs as they leave the ribosome (Rose et al. 2006). However, this self-assembly is not always energetically favoured and therefore folding catalysts (HSPs) are required to overcome this barrier (Balchin et al. 2016). All HSPs that facilitate protein folding, utilise intimate protein–protein contacts to firstly stabilise the unfolded peptide chain and then reduce the energy of the folding transition states (analogous to all enzymes) of the target protein (Balchin et al. 2016). Members of the HSP70 family complete this task via a common, highly conserved modular domain structure comprising: an N-terminal nucleotide binding domain (NBD)/ATPase domain, a centrally located linker region, and a substrate-binding domain (SBD) located within the C-terminus (Mayer and Bukau 2005) (Fig. 1a). Such structural features underpin the chaperoning function of the HSP70 proteins by

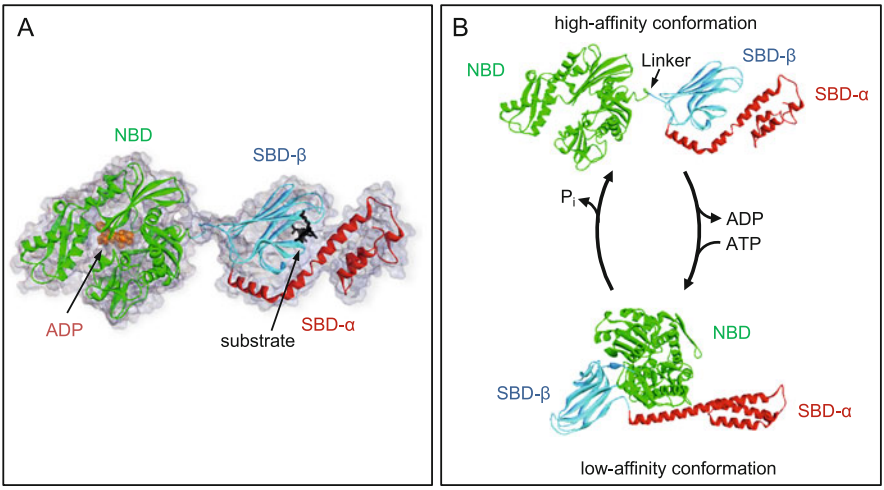

Fig. 1 Structure and reaction cycle of HSPA2. (**a**) Members of the HSP70 family, including HSPA2, share a highly conserved modular domain structure comprising an N-terminal nucleotide binding domain (NBD)/ATPase domain, a centrally located linker region, and a substrate-binding domain (SBD) that is located within the C-terminus and comprised of a beta-sandwich subdomain (SBD-β) and an alpha-helical lid region (SBD-α). (**b**) The HSPA2 structure alternates between two conformations. The high-affinity conformer is stabilised when ATP is hydrolysed leaving ADP bound to the NBD. Once substrate folding is complete, ADP is released allowing ATP to bind. This causes the protein to undergo a conformational change to the low-affinity form. All structures are HSP70 homology models based on pdb: 2KHO (Bertelsen et al. 2009) and pdb: 4B9Q (Kityk et al. 2012)

enabling them to bind extended stretches of hydrophobic amino acids, exposed within incorrectly folded proteins in an ATP-dependent manner. The C-terminus SBD contains the least conserved primary sequences and, together with divergent expression profiles and localisation within specific subcellular compartments (ranging from the ER lumen, mitochondrial matrix, cytosol, and nucleus), are able to afford a degree of specificity in terms of the spectrum of client protein interactions and thus contribute to the nonredundant functions of HSP70 family members.

2.3 Structural Features of HSPA2

In order to understand the multifaceted function of HSPA2 in the context of male germ cell development and function, we contend that it is critical to first understand the structural architecture of this molecule. While many aspects of the HSP70 structure are understood, subtleties including how HSPA2 interacts with external client proteins and facilitates multimeric complex formation remain to be defined. As is the case for all HSP70s, the NBD and SBD within HSPA2 are joined by a conserved hydrophobic linker domain (Fig. 1a). Structurally, the NBD comprises two lobes, each forming two subdomains, giving rise to the nucleotide-binding cleft situated in between. This domain has a strong affinity for adenosine nucleotides and once bound, can be utilised to drive its conformational cycle (Fig. 1b). The SBD is comprised of a beta-sandwich subdomain (SBD-β) and an alpha-helical lid region (SBD-α) (Fig. 1a). The SBD is able to bind peptide residues consisting of 5–7 amino acids enriched in hydrophobic sidechains. Hydrophobic peptides that are flanked with positively charged residues elevate the binding efficiency of the SBD (Rudiger et al. 1997). This hydrophobic peptide profile is common to many proteins, but the degree to which it is exposed increases in denatured or newly translated proteins.

A combination of hydrogen bonds that form between the SBD and the substrate peptide backbone as well as van der Waals contacts with hydrophobic side chains mediates peptide binding in an extended conformation and facilitates the stability of these transient species before they are either folded or subjected to further processing. The molecular function of HSPA2 relies on the conformational changes triggered by ATP hydrolysis in the NBD, which in turn regulates peptide binding and release. When ATP binds to the NBD, it results in the attachment of the hydrophobic interdomain linker and the alpha-helical lid of the SBD to the NBD, which opens the peptide binding pocket in the SBD (Fig. 1b). At this point, the conformation of HSP70s is referred to as an 'open state'. This conformation allows rapid 'on and off' interactions with HSP70 substrates exhibiting a low substrate affinity and is responsible for the stabilisation of new or denatured proteins, preventing the aggregation of these unfolded polypeptides (Fig. 1b). The promotion of protein folding (in conjunction with co-chaperones), the mediation of multimeric complex formation, or the facilitation of target protein translocation across membranes is then initiated by ATP hydrolysis. The resultant formation of ADP within

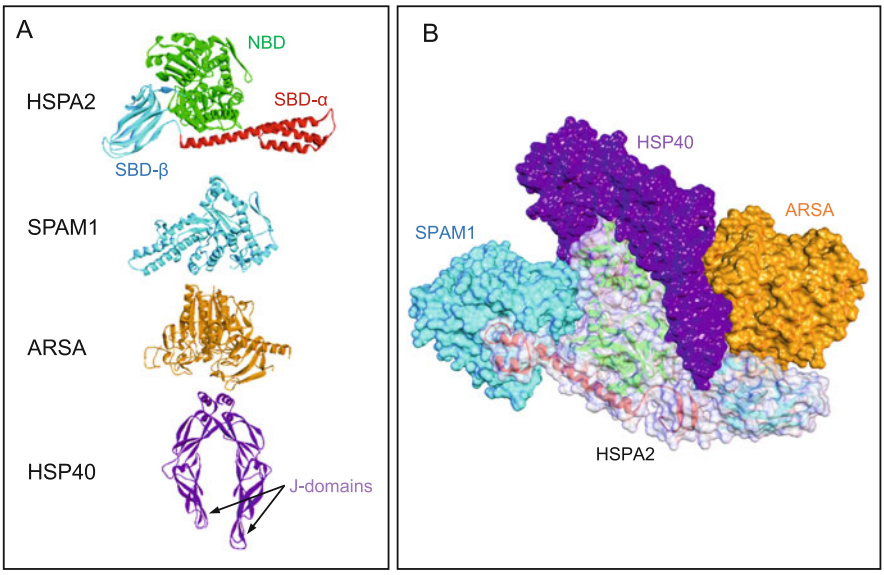

Fig. 2 Structural model of a putative HSPA2 oocyte receptor complex. (**a**) HSPA2 has been implicated in the assembly of multimeric protein complexes that putatively act as oocyte receptors in mature human spermatozoa. The most well characterised of these comprises HSPA2 in association with sperm adhesion molecule 1 (SPAM1) and arylsulfatase A (ARSA). Structures and homology models of HSPA2, HSP40 (DNAJB1), SPAM1, and ARSA are depicted. (**b**) Putative quaternary structure of the hetero-oligomeric oocyte–receptor complex formed from the HSPA2, HSP40 (DNAJB1), SPAM1, and ARSA subunits

the NBD leads to the release of the lid from the NBD as well as the capture of the target peptide within the SBD, thus leading to low on and off rates and promoting a high substrate affinity. At this point, the conformation is recognised as being in a 'closed state' and under such an arrangement, the SBD and NBD are loosely held together by the linker (Frydman 2001) (Fig. 1b). This entire reaction cycle proceeds in an analogous manner among most members of the HSP70 family (Mayer and Kityk 2015) (Fig. 2b).

The velocity of ATP hydrolysis and therefore its activity is regulated by several factors but can be primarily increased by substrate binding, which is in turn strongly influenced by HSP40 co-chaperone proteins. The critical partnerships formed with HSP40s (or DNAJ) are key to the regulation of HSP70 refolding activity (Kampinga and Craig 2010; Laufen et al. 1999) and are also known to influence the organisation of HSP70 quaternary structures, thus promoting the formation of either homodimers, oligomers or hetero-oligomeric species (Morgner et al. 2015; Sarbeng et al. 2015; Aprile et al. 2013). Acquired substrates that are not completely processed or unable to fold rapidly upon dissociation from HSP70 may rebind allowing several refolding cycles facilitated by class I and class II HSP40s (see Sect. 2.5) or alternatively transfer to downstream heat shock proteins such as

HSP90. If the peptide or protein is unlikely to conform to its native geometry, it can be then be transferred to the protein degradation machinery (Shiber and Ravid 2014).

The flexible and modular nature of HSPA2 offers enormous plasticity in its structure/function and may account for the range of tasks in which it is involved. While this flexibility also ensures that gaining an understanding of the mechanisms that drive HSPA2s specific functions is a challenging task, structural and kinetic studies remain critical for furthering our understanding of this cellular machine.

2.4 The Importance of HSPA2 Complexation

There is emerging evidence that HSP70 family members may exist as oligomers (Aprile et al. 2013). For conceptualisation purposes, the crystal structure for a bacterial homologue SSE1 (pdb:2QXL) (Liu and Hendrickson 2007) has been resolved as a dimer, which is also of some functional significance for refolding in the HSP network with HSP40 (Sarbeng et al. 2015) and HSP90 (Morgner et al. 2015) (themselves also existing in dimeric form). It has been documented that the HSP70 linker region facilitates the construction of homo-oligomers that serve as a reservoir of protein under low stress levels (Aprile et al. 2013). Upon interaction with a threshold concentration of a substrate, the oligomers breakdown to yield active monomeric units. This substrate-driven equilibrium may offer another regulatory mechanism for engaging heat shock protein activity when required. While such interactions outline the propensity of HSP70 molecules to promote the formation of multimeric complexes, the consensus, however, clearly indicates that the protein refolding tasks are directed by the monomeric entity. Additional supporting evidence that precludes monomers as the only important HSP70 species has been provided by the demonstration that HSP70 dimerisation, working in concert with the HSP40 J-domain, assists in substrate transfer to HSP90s (Morgner et al. 2015). Further, a recent study using targeted mutagenesis of the key residues that facilitate dimerisation of the bacterial DNAK (a HSP70 homologue) has reported a concomitant loss of protein function (Sarbeng et al. 2015). While the existence of such data confirm the functional importance of HSP70 quaternary structure, the mechanisms(s) by which the protein orchestrates the formation of these functional complexes, particularly the hetero-oligomeric species implicated in sperm function (see Sect. 3), remains poorly understood. Nevertheless, as a means of illustrating how such interactions may be driven, the following section reviews the structural and functional properties of the HSP40 co-chaperones that are known to target HSP70s to their substrates and fine-tune the resultant binding and release cycles that dictate the longevity of HSP70–substrate complexes.

2.5 HSP40 Co-Chaperone Regulation of HSP70 Function

HSP40 proteins (also known as J-proteins) operating in the capacity of co-chaperones (Kim et al. 2013b) rank among the principle regulators of HSP70 function in mammalian species (Ohtsuka and Hata 2000). In this capacity, HSP40s act by modifying HSP70 ATPase activity, thus strongly increasing the rate of ATP hydrolysis within the NBD. The consequent increase in bound ADP results in conformational changes that drive HSP70 refolding activity as detailed previously (Sect. 2.3, Fig. 1a). On the basis of distinctive structural features, the HSP40 proteins are generally divided into three subtypes, namely: classes I–III. The principle domains of HSP40s are the J-domain, the G/F-rich region, zinc-finger-like motifs, and a conserved C-terminal peptide binding domain. Class I HSP40s, such as HDJ2, consist of an N-terminal J-domain, a G/F-rich region, two zinc-finger-like motifs, and a conserved C-terminal peptide-binding domain (CTD) (Kim et al. 2013b). Class II HSP40 proteins, such as HDIJ1, are similar to class I proteins, except that both zinc-finger-like motifs are replaced with G/F-rich regions (Perales-Calvo et al. 2010; Kim et al. 2013b). Although both class I and II HSP40s directly bind and modulate the capacity of HSP70s to bind nonnative substrate proteins, the mechanisms behind the regulation are dissimilar (Kim et al. 2013b). Thus, class I HSP40s are able to suppress peptide aggregations independently, while class II HSP40s are reliant on the partnering HSP70 to suppress peptide aggregation (Frydman 2001; Frydman 2001). Similarly, class I and II HSP40s interact with HSP70 via docking of the J-domain to the HSP70 NBD, while the CTD is responsible for recognition of the HSP70 C-terminus (Suzuki et al. 2010). The remaining class III HSP40s comprise a more diverse range of HSPs and primarily connect the J-domain with various other functional modules (Frydman 2001). Overall, in excess of 40 HSP40 proteins have been identified within the human proteome, compared with fewer than 20 HSP70s. Such redundancy suggests that more than one HSP40 is capable of regulating each HSP70 (Kim et al. 2013b) and may offer an additional explanation to account for the diversity of HSP70 functions.

Of particular interest in the context of this chapter is the identity of the HSP40 isoform(s) that are present and functional in the male germline. Interestingly, several HSP40s (e.g. DNAJA1, DNAJA2, DNAJB1, DNAJB3, DNAJB13) have been implicated in the regulation of testicular germ cell development with diverse functions extending from the promotion of germ cell progression, apoptosis, androgen signalling, sperm tail formation, and the acquisition of fertilisation potential (Berruti and Martegani 2001; Berruti et al. 1998a, b; Guan and Yuan 2008; Hafizur et al. 2004; Hu et al. 2004; Li and Liu 2014; Meccariello et al. 2002, 2014; Terada et al. 2005; Yang et al. 2005a, b; Yu and Takenaka 2003; Doiguchi et al. 2007). Analysis of transgenic mouse models has demonstrated that the targeted ablation of DNAJA1 induces severe deficits in spermatogenesis that appear inextricably linked to aberrant androgen signalling (Terada et al. 2005). Notably, the critical role of DNAJA1 in spermiogenesis was not able to be compensated for by the presence of

the related DNAJA2 protein, suggesting that these HSP40 isoforms are not functionally equivalent in testicular tissue. Similarly, the lack of DNAJA1 expression did not influence the testicular expression of HSPA2 in these mice, indicating that it is unlikely to act as a co-chaperone for HSPA2. Rather, this role may fall to a mouse HSP40 homologue referred to as MSJ-1, which is abundantly expressed in the adult testis (Berruti and Martegani 2001). Specifically, MSJ-1 expression levels appear to be developmentally regulated with notable increases coinciding with the formation of differentiating spermatids. In these germ cells, the MSJ-1 protein co-localises with the developing acrosomal and post-nuclear domains of the sperm head (Berruti and Martegani 2001), a subcellular localisation that mirrors that reported for HSPA2 (Sect. 3.1) (Berruti and Martegani 2001). MSJ-1 is also retained in epididymal spermatozoa, where it localises to the outer surface of the acrosomal vesicle and to the centrosomal region (Berruti and Martegani 2001). In support of its co-chaperoning role, MSJ-1 is able to interact with HSPA2 and can be co-immunoprecipitated with the protein from spermatogenic cells, although information regarding the specific substrate or client proteins associated with this HSP70–HSP40 partnership remains unresolved.

2.6 *BAG6 Co-Chaperone Regulation of HSP70 Function*

Another critical regulator of HSP70 function is BCL2-associated athanogene 6 (BAG6; formerly BAT3/Scythe). BAG6 is a multifunctional chaperone of the BAG-domain protein family (Lee and Ye 2013). Though initially characterised as a part of the major histocompatibility complex III (Banerji et al. 1990), its ascribed roles now extend to apoptotic regulation, protein synthesis, protein quality control, and degradation (Desmots et al. 2005; Minami et al. 2010; Shaha et al. 2010; Thress et al. 2001). The latter event is mediated by its N-terminal ubiquitin-like (UBL) domain while its C-terminal 'BAG-like' domain affords it the ability to interact with the ATP-binding site of HSP70 family proteins (Kuwabara et al. 2015; Thress et al. 2001). This interaction promotes the release of HSP70-bound substrates and hence allows for the diversification of HSP70 cellular functions. Evidence for the post-translational regulation of HSP70 by BAG6 stems from studies evaluating both its importance for HSP70 accumulation upon heat shock in fibroblast cells (Corduan et al. 2009) and its regulation of HSPA2 stability in developing germ cells (Sasaki et al. 2008). In the context of spermatogenesis, BAG6 has been shown to interact with HSPA2 and prevent its ubiquitination by hindering the access of ubiquitin ligases (Sasaki et al. 2008). However, in *Bag6*-deficient germ cells, HSPA2 is rapidly degraded by the ubiquitin-proteasome system leading to impaired spermatogenesis and male infertility (Sasaki et al. 2008), both hallmarks of *Hspa2*-deficiency in the testis (Sect. 3.1). Furthermore, the depletion of *Bag6* from either mouse embryonic fibroblasts or human teratocarcinoma cells also results in decreased HSPA2 stability, thus implying that this BAG6-dependent mechanism may be a highly conserved system for HSPA2 regulation (Sasaki et al. 2008).

3 The Role of Hspa2 in Male Germ Cell Development and Function

3.1 The Role of HSPA2 in Mouse Germ Cell Development

In the following sections, we consider the evidence that HSPA2 provides a nucleus for the assembly of multimeric protein complexes that regulate germ cell development and function. The majority of work defining the role of HSPA2 in germ cell development has focused on the mouse model. The original identification of HSPA2 in this species stems from experiments in which the proteome of the male germ line was assessed following the induction of heat stress (Allen et al. 1988a, b; O'Brien 1987). Such an insult is particularly pertinent to a majority of mammalian species, including our own, owing to the adaptation of testicular descent into a scrotum that is typically maintained at temperatures of between 2 and 7 °C below that of the core body temperature (Waites 1991). Although the adaptive significance of the cooler environment afforded by the scrotum is still being actively debated, it has been proposed that this temperature differential maintains optimal spermatogenesis, minimises gamete mutation rates, and/or supports sperm maturation and storage in the epididymis [reviewed by (Gallup 2009)]. While heat shock proteins constitute a key element of the cell stress response mounted to ameliorate the detrimental impact of such insults (Kim et al. 2013a), it is notable that the expression of the mouse *Hspa2* gene is not induced following heat shock as may be expected of proteins involved in buffering spermatogenesis against thermal injury (Widlak et al. 2007; Zakeri et al. 1990). In fact, the opposite is true with elevation of testicular temperature (e.g. by experimental cryptorchidism) leading to a rapid loss of *Hspa2* mRNA and a concomitant deterioration of the seminiferous epithelium (Kon and Endoh 2001). Notably, homologous *Hspa2* mRNA transcripts expressed in both rat (Krawczyk et al. 1987) and rhesus monkey testes (Zhou et al. 2001) have also been reported to be vulnerable to factors, such as heat, that compromise spermatogenesis. This behaviour contrasts that of other members of the HSP70 family, whose expression profiles are dramatically increased following testicular exposure to either acute (Cao et al. 2009) or chronic heat stress (Pei et al. 2012).

Despite the absence of a heat inducible expression pattern, several lines of evidence implicate HSPA2 in the regulation of male reproductive potential. For instance, *Hspa2* mRNA transcripts, and the protein itself, display significant enrichment within testicular tissue where they are characterised by a developmentally regulated expression profile (Dix et al. 1996b; Murashov and Wolgemuth 1996; Rosario et al. 1992; Zakeri et al. 1988). Thus, *Hspa2* gene expression is first recorded during the early phases of meiosis I (Dix et al. 1996b; Rosario et al. 1992) commensurate with the detection of HSPA2 protein synthesis in leptotene–zygotene spermatocytes (Dix et al. 1997). Although the precise nature of the factors responsible for regulating this gene expression profile remains to be definitely established, it has been postulated that they include positive influences imposed by testis-specific transcription factors and epigenetic elements, combined with the

reciprocal action of negative repressors during the preceeding phases of spermatogonial and early spermatocyte development [reviewed by Scieglinska and Krawczyk (2014)]. Irrespective of this, an indispensable role for the HSPA2 protein in supporting the transition of spermatogenic cells through the meiotic stages of spermatogenesis has been revealed through selective ablation of the *Hspa2* gene (Mori et al. 1997).

The resultant infertility phenotype of *Hspa2* null males has been attributed to the combined effects of arrested spermatogenic cell development coinciding with the G_2–M-phase transition of prophase of meiosis I, as well as the apoptotic elimination of late stage pachytene spermatocytes (Allen et al. 1996; Mori et al. 1997). In accounting for such defects, at least two primary roles have been postulated for the HSPA2 protein within developing germ cells, both of which appear to rely on the ability of HSPA2 to promote the formation of multimeric protein complexes. In the first instance, HSPA2 appears to facilitate the interaction between cyclin-dependent kinase 2 (CDC2) and the regulatory cyclin B1 (Zhu et al. 1997), and in the second, HSPA2 is proposed to function as a modulator of the synaptonemal complex (Mori et al. 1997) that forms between homologous chromosomes to mediate chromosome pairing, synapsis, and meiotic recombination events. Specifically, HSPA2 appears necessary for synaptonemal complex desynapsis and thus the completion of meiosis I in spermatocytes.

In addition to its support of meiosis, HSPA2 has also been implicated in the chaperoning of spermatid-specific DNA packaging transition proteins in post-meiotic male germ cells (Govin et al. 2006). In this capacity, HSPA2 appears to escort the intermediary transition proteins TP1 and TP2 to the paternal genome prior to facilitating their assembly into DNA packaging structures (Govin et al. 2006) that promote nuclear compaction. In a similar context, the chaperoning activity of HSPA2 has also been causally linked to the correct folding, assembly, and trafficking of the subunits comprising the CatSper ion channel, which is required for sperm cell hyperactivation and male fertility (Liu et al. 2007).

It has recently become apparent that the various functions assigned to HSPA2 during germ cell development may be fine-tuned via its interaction with an additional subset of testis-enriched proteins, including Shc SH2 domain-binding protein 1-like protein (SHCBP1L) (Liu et al. 2014), the nuclear autoantigenic sperm protein (tNASP) (Alekseev et al. 2009), and the DExD-box helicase MOV10-like-1 (MOV10L1) that holds an essential role in safeguarding the paternal genome (Frost et al. 2010). As discussed previously (Sect. 2.6), the stability of the HSPA2 protein during germ cell development is also profoundly influenced by its interaction with BAG6, with the loss of this co-chaperone leading to the poly-ubiquitination and subsequent degradation of the protein (Sasaki et al. 2008).

3.2 The Role of HSPA2 in Germ Cell Development of Non-rodent Species

In addition to its characterisation in mouse testes, a homologue of the HSPA2 protein has also been identified in human testes thus raising the prospect that it may hold a conserved functional role in the support of spermatogenesis (Eddy 1999). Consistent with this notion, the degree of amino acid sequence conservation of the human and mouse HSPA2 homologues exceeds 98% (Bonnycastle et al. 1994). However, while the HSPA2 protein has proven to be abundantly, and selectively, expressed in the developing germ cells within human testes (Son et al. 1999), its expression profile and potential function do not strictly mirror those described in the mouse. Thus, HSPA2 appears to be expressed in a biphasic pattern during human spermatogenesis, with the protein first appearing in spermatocytes where it is predicted to support meiosis (Huszar et al. 2000). A second wave of expression is subsequently initiated during the formation of elongating spermatids that accompanies spermiogenesis (Huszar et al. 2000). In this latter role, HSPA2 has been implicated in cytoplasmic extrusion and remodelling of the plasma membrane architecture (Huszar et al. 1997, 2006).

At present, there is no evidence to support analogous roles for human HSPA2 in regulating desynapsis of the synaptonemal complex or in the chaperoning of cyclin–CDK complexes as has been reported in the mouse. Nevertheless, the importance of the protein for human male germ cell development is evidenced by the identification of a strong correlation between the level of testicular expression of the *Hspa2* gene and that of ejaculate sperm concentration. Indeed, *Hspa2* gene expression has been reported to be significantly reduced in male infertility patients afflicted with either oligozoospermia (i.e. low sperm concentration) (Cedenho et al. 2006) or complete non-obstructive azoospermia (i.e. no detectable spermatozoa within an ejaculate) arising from either spermatocyte arrest or Sertoli cell-only syndrome (Son et al. 2000). Underrepresentation of the HSPA2 protein has also been reported in the proteome of spermatozoa from infertile men with clinical varicocele (Agarwal et al. 2016; Lima et al. 2006), a pathology attributed to abnormal dilation of the testicular veins in the pampiniform venous plexus. Similarly, levels of HSPA2 are dysregulated in the proteomic signature of spermatozoa from infertile males diagnosed with asthenozoospermia (defined as the production of semen samples with $<25\%$ progressive sperm motility or $<50\%$ motile sperm). However, in contrast to the reduced HSPA2 expression associated with most other forms of infertility, HSPA2 levels are actually increased in the spermatozoa of asthenozoospermic patients (Martinez-Heredia et al. 2008).

Taken together, such data clearly identify the HSPA2 protein as being particularly vulnerable to a range of acute and chronic stressors, and that the resultant loss and/or dysfunction of the protein that follows exposure to such insults has direct and profound consequences for male fertility. The potential for evolutionary conservation of such function(s) is suggested by the identification of *Hspa2* gene and/or immunoreactive protein homologues in the testis (or testis homogenates) from a

range of metazoan species extending from mammals (e.g. mouse, rat, human, guinea pig, dog, pig, bulls, rabbit) through to phyla as diverse as birds (e.g. chickens), amphibians (Xenopus laevis), and fish (cod) [reviewed by Eddy (1999)]. While further experimental work is clearly necessary prior to assigning equivalent functions to the HSPA2 homologues identified across these species, it is perhaps notable that the *Hspa2* nucleotide and HSPA2 amino acid sequences share a relatively high degree of identity (Padhi et al. 2016). Indeed, recent phylogenetic analysis focusing on the ratio of non-synonymous (amino acid replacement) to synonymous (no change in amino acid) nucleotide mutations has provided evidence that the primary structure of HSPA2 homologues has been constrained by intense negative (or purifying) selection, particularly within the mammalian lineage (Padhi et al. 2016). Such circumstances lend support to the notion of conserved HSPA2 function.

Notably however, this situation contrasts that reported in avian species wherein HSPA2 appears to display signatures of a unique evolutionary trajectory. Thus, avian HSPA2 homologues exhibit sequence divergence bearing the hallmarks of positive (adaptive) selection. This is particularly true of the peptide-binding domain occupying the c-terminus of the protein (Padhi et al. 2016). Such changes are likely to impose significant structural and thus functional consequences on the HSPA2 protein and accord with distinctive responses of the protein to heat stress in mammalian germ cells versus that of their avian counterparts. Indeed, in contrast to the mammalian system where testicular *Hspa2* transcripts are rapidly depleted upon induction of heat stress (Sect. 3.1), an opposing response has been documented in the rooster with elevated testicular temperature leading to a concomitant up-regulation of *Hspa2* gene expression (Wang et al. 2013). On the basis of these combined data, it has been argued that HSPA2 may constitute a key element of the adaptive mechanism(s) required to accommodate efficient spermatogenesis at the elevated internal temperature of ~40–41 °C (Beaupre et al. 1997) that the testes are exposed to within the body cavity of birds (Padhi et al. 2016; Mezquita et al. 1998).

3.3 The Role of HSPA2 in Human Sperm Function

In addition to the aforementioned studies implicating HSPA2 as holding fundamental role(s) in ensuring the fidelity of germ cell development within the testes, the fact that the protein is retained in spermatozoa raises the prospect that HSPA2 may fulfil ancillary roles in the mature gamete (Bromfield and Nixon 2013; Dun et al. 2012). While there is a current dearth of experimental evidence exploring this possibility in animal models such as the mouse, the prospect that HSPA2 takes on specialised roles in mature spermatozoa is supported by mounting evidence from studies conducted in our own species. Indeed, as described previously (Sect. 3.2), reduced HSPA2 protein expression has been reported in immature human spermatozoa that appear to have failed to complete normal spermiogenesis (Ergur et al. 2002). Such a defect not only manifests in the production of spermatozoa with

excessive cytoplasmic retention (Huszar et al. 1997, 2000) but also cells that harbour a reduced ability to penetrate the cumulus matrix and bind the zona pellucida (ZP) surrounding the ovulated oocyte (Ergur et al. 2002; Huszar et al. 2006). Such lesions have been attributed to a loss of the HSPA2 chaperoning activity and a commensurate dysregulation of plasma membrane remodelling during spermiogenesis (Ergur et al. 2002; Huszar et al. 2000, 2003, 2006; Kovanci et al. 2001). These critical events are, in turn, necessary for establishing the formation of the ZP-binding domains as well as those required for penetration of the hyaluronic acid-rich matrix of the cumulus mass. It follows that relative amount of HSPA2 present in mature human spermatozoa provides a strong discriminative index of fertilising potential (Ergur et al. 2002; Huszar et al. 2006, 2007).

More recently, such defects have been corroborated through comparative proteomic analyses in which HSPA2 has been independently identified as being underrepresented in the spermatozoa of infertile patients harbouring an isolated lesion in oocyte recognition (Redgrove et al. 2012). An important caveat, however, is that the latter study failed to link HSPA2 deficit with accompanying defects in either sperm motility or morphology. This evidence has fuelled the proposal that HSPA2 may facilitate the functional maturation of spermatozoa after the completion of their morphological differentiation within the testes (Bromfield and Nixon 2013; Nixon et al. 2015). In support of this model, HSPA2 localises within the peri-acrosomal domain of mature human spermatozoa, a position compatible with regulation of oocyte interactions (Redgrove et al. 2012). At present, there remains some conjecture as to whether HSPA2 is constitutively expressed on the surface of the human sperm plasma membrane (Naaby-Hansen and Herr 2010), undergoes an increase in surface expression following the induction of capacitation (Motiei et al. 2013), or resides exclusively within an intracellular location (Redgrove et al. 2012, 2013). Such contention may, at least in part, reflect the use of different methods of protein detection and/or antibodies (Scieglinska and Krawczyk 2014). Irrespective, on the basis of available evidence, it seems likely that HSPA2 fulfils an indirect role in mediating sperm–egg recognition (Redgrove et al. 2012). Specifically, we posit that HSPA2 facilitates the assembly, repositioning and/or unmasking of ZP recognition complexes on the sperm surface (Nixon et al. 2011; Redgrove et al. 2012, 2013). This supposition accounts for the demonstration that HSPA2 stably interacts with a number of multimeric complexes, with aggregate molecular weights in excess of 150 kDa, in the spermatozoa of fertile normozoospermic individuals (Bromfield et al. 2015a, 2016; Redgrove et al. 2011, 2012). Notably, this putative mechanism of heat shock protein-mediated remodelling of the maturing sperm cell also draws support from studies conducted in the mouse (Dun et al. 2010, 2012; Nixon et al. 2005, 2007). Curiously however, it appears that distinct heat shock proteins may have been recruited for this purpose in different species, with members of the HSP60 (HSPD1), HSP90 (HSP90B1), and chaperonin family each being implicated as fulfilling equivalent roles in mouse spermatozoa (Asquith et al. 2004; Dun et al. 2011).

In studies focusing on the characterisation of the molecular composition of the predominant HSPA2 complexes, we have identified several putative client proteins

among the human sperm proteome (Bromfield et al. 2015a, 2016; Redgrove et al. 2012). The most well studied of these complexes comprises HSPA2 and an additional two proteins, sperm adhesion molecule 1 (SPAM1) and arylsulfatase A (ARSA) (Fig. 2), both of which have been implicated in interactions with the cumulus–oocyte complex (Xu et al. 2012; Wu et al. 2007; Tantibhedhyangkul et al. 2002; Carmona et al. 2002; Zhou et al. 2012; Martin-Deleon 2011; Kimura et al. 2009). The components of this complex appear to undergo a marked, capacitation-associated remodelling that culminates in ARSA being repositioned such that it becomes accessible to antibody labelling on the outer leaflet of the sperm surface, a location compatible with a role in the mediation of sperm–ZP interactions (Redgrove et al. 2012). Conversely, SPAM1 appears to reorient such that its surface detection is reduced, possibly reflecting its primary role in cumulus matrix dispersal preceding sperm–ZP recognition (Redgrove et al. 2012). In addition to aligning with the functional requirements of the fertilising spermatozoon, this regulated shift in protein accessibility is compatible with the observation that spermatozoa in the advanced stages of capacitation lose their ability to bind hyaluronic acid (Cayli et al. 2004).

We have recently extended these observations to reveal that dynamic, capacitation-associated repositioning of human sperm proteins includes additional HSPA2 client proteins located in the peri-acrosomal region of the sperm head. Specifically, we have identified an HSPA2 assemblage comprising the novel interacting partners of angiotensin converting enzyme (ACE) and protein disulphide isomerase A6 (PDIA6) (Bromfield et al. 2016). Interestingly, the accessibility to surface labelling of PDIA6, but not ACE, is influenced by the sperm capacitation status. In terms of the functional significance of this protein complex, pharmacological inhibition of ACE significantly reduced the ability of human spermatozoa to undergo an agonist induced acrosome reaction (Bromfield et al. 2016). Taken together, these data raise the possibility that HSPA2 holds a key role in priming the sperm surface architecture in advance of their interaction with the cumulus–oocyte complex (Bromfield and Nixon 2013). Furthermore, we have recently shown that the HSPA2-regulatory chaperone BAG6 also appears to be incorporated into mature human spermatozoa where it forms a stable complex with HSPA2 and ADAM30 in the apical region of the sperm head (Bromfield et al. 2015a). Although a role for BAG6 in mature spermatozoa is yet to be defined, we postulate that the presence of BAG6 may be important in the prevention of protein misfolding or mislocalisation events during HSPA2-dependent assembly of zona pellucida receptor complexes at the sperm surface (Bromfield et al. 2015a). In this way, the presence of BAG6 may ensure correct functioning of HSPA2 prior to sperm–oocyte interaction. Moreover, our preliminary analyses of BAG6 in the infertile patient population has revealed that spermatozoa that lack the ability to interact with homologous human zona pellucidae, associated with dysregulation of HSPA2 protein expression, also have a severe deficiency in BAG6 protein expression compared with fertile controls (Bromfield et al. 2015a). Such findings provide an interesting link between the underrepresentation of HSPA2 in the patient population and its regulation by co-chaperones.

At present, it remains uncertain how heat shock protein-mediated remodelling of the sperm surface is achieved and whether it involves the simple unmasking of epitopes or more complex repositioning of client proteins. Nevertheless, it is noteworthy that HSPA2 complexes appear to colocalise with structural markers of membrane rafts, microdomains that themselves have been shown to be repositioned within the sperm head during capacitation (Nixon and Aitken 2009; Nixon et al. 2011). Furthermore, we have shown that capacitation-driven post-translational modification of HSPA2 is of critical importance, such that HSPA2 client protein repositioning can be abrogated by incubation of spermatozoa in broad spectrum tyrosine kinase inhibitors (Redgrove et al. 2013). Interestingly, exposure of mature sperm to oxidative stress can also compromise HSPA2-mediated remodelling of the sperm surface architecture (Fig. 3) (Bromfield et al. 2015b).

4 Mechanisms Underpinning the Loss of Hspa2 from the Spermatozoa of Infertile Patients

A key unresolved question in the field of male germ cell development and function is how the expression of the HSPA2 protein is selectively dysregulated in the spermatozoa of a subset of infertile men. As we have previously noted (Nixon et al. 2015), there are numerous possibilities that could account for the attenuation of HSPA2 levels in these defective sperm populations. Foremost among these are mutations in the encoding *Hspa2* gene, altered epigenetic regulation of *Hspa2* gene expression, and/or perturbations in protein expression/stability arising from exposure of developing germ cells to oxidative stress. While the former explanations cannot be discounted [reviewed by Scieglinska and Krawczyk (2014)], our favoured model currently centres on the detrimental impact of oxidative stress (Fig. 3), an insult that is commonly, and causally, associated with the aetiology of male infertility (Aitken et al. 2014).

Among the conserved features of oxidatively stressed spermatozoa, is the induction of a lipid peroxidation cascade that culminates in the formation of a range of small molecular mass aldehydes that are capable of forming adducts with both nucleotides and proteins. In recent studies, it has been shown that one of the most commonly formed compounds, 4-hydroxynonenal (4HNE), will adduct to proteins localised within the head and midpiece of human spermatozoa (Aitken et al. 2012; Bromfield et al. 2015b; Moazamian et al. 2015). Through the targeting of mitochondrial proteins localised in the latter location, 4HNE is able to elicit a pathophysiological response characterised by elevated electron leakage from the electron transport chain, a concomitant loss of mitochondrial membrane potential, oxidative DNA damage, and eventually cell death (Aitken et al. 2012). In contrast, the accumulation of 4HNE adducts in the sperm head are able to compromise sperm–oocyte recognition and adhesion independent of cytotoxicity and/or motility defects (Bromfield et al. 2015b). In recent studies, we have provided evidence that

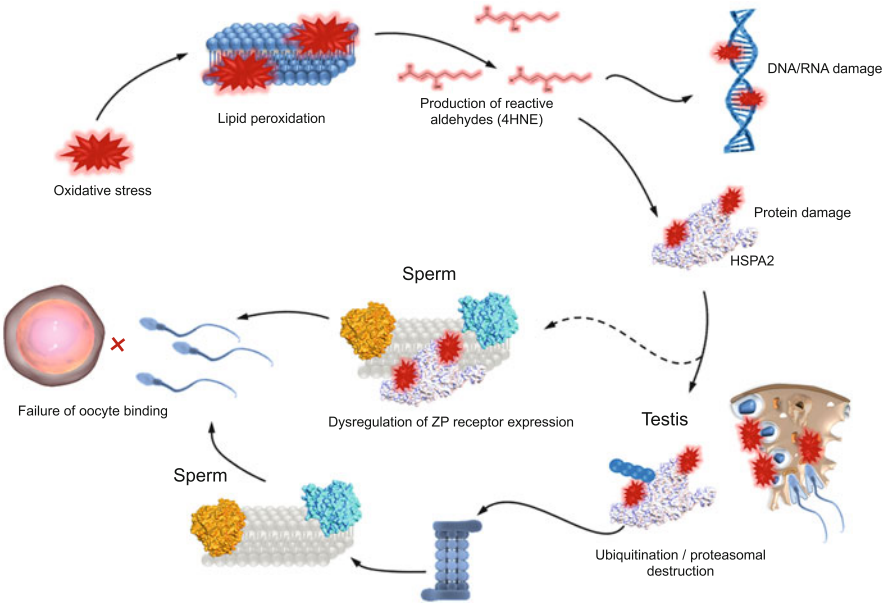

Fig. 3 Putative mechanism underpinning the loss of heat shock protein A2 (HSPA2) from the spermatozoa of infertile patients. Although the precise mechanism(s) underpinning the selective loss of HSPA2 from the differentiating gametes of infertile patients is currently unknown, our favoured hypothesis centres on perturbations in protein expression/stability arising from exposure of developing germ cells to oxidative stress. In this model, it is postulated that a localised elevation in reactive oxygen species could precipitate a lipid peroxidation cascade leading to the production of highly reactive lipid aldehydes such as 4-hydroxynonenol (4HNE). While such products have the capacity to damage the *Hspa2* gene and/or mRNA transcripts, we have shown that HSPA2 protein itself is directly targeted for adduction by 4HNE. Further, this insult can dysregulate the assembly of HSPA2 oocyte receptor complexes in mature spermatozoa leading to a loss of their ability to recognise the oocyte. If such an oxidative attack were to strike earlier during germ cell development, we posit that this could lead to ubiquitination of the HSPA2 protein and thus accelerate its proteolysis through proteasomal (or lysosomal) pathways. The ensuing loss of the HSPA2 protein from the mature sperm proteome would prevent the correct positioning of oocyte receptors and also account for the loss of oocyte recognition

such a response is due, at least in part, to the dysregulation of HSPA2 function (Bromfield et al. 2015b) brought about by selective 4HNE adduction of the protein (Baker et al. 2015). Specifically, we have shown that the 4HNE modification is likely to cause an attenuation of HSPA2 chaperone activity and a concomitant dysregulation of its ability to remodel the sperm surface in preparation for ZP binding (Bromfield et al. 2015b) (Fig. 3). Such dysregulation extends to not only the predominant HSPA2/SPAM1/ARSA receptor complex, but also the HSPA2/PDIA6/ACE complex we have characterised in human spermatozoa (Bromfield et al. 2015b, 2016). The causal nature of this response is evidenced by the ability to at least partially ameliorate the detrimental impact of 4HNE through the use of penicillamine to reduce the bioavailability of this aldehyde. Taken together, these

data suggest that heat shock proteins are key targets for 4HNE adduction reactions. Given that their chaperoning activity is governed by nucleotide affinity and intimate protein–protein interactions with substrates, the acquisition of significant structural modifications in the NDB and/or SBD precipitated by 4HNE adduction renders HSPA2 particularly susceptible to such an insult.

In terms of its mechanism of action, 4HNE adduction is known to preferentially target cysteine, lysine, and histidine residues and thus elicit a number of structural and functional changes including a loss of substrate recognition or refolding capacity (Butterfield and Lauderback 2002; Esterbauer et al. 1991; Perluigi et al. 2012; Uchida 2003; Uchida and Stadtman 1993). In the case of HSPA2, 4HNE has been shown to covalently modify the Cys267 residue residing within the NBD of the molecule (Carbone et al. 2004). Acting through this purported thiol-specific mechanism, adduction is thereby likely to attenuate the ATPase activity of HSPA2 and prevent it from fulfilling its role in protein trafficking and/or refolding events (Carbone et al. 2004, 2005). Interestingly, an analogous model of 4HNE-driven thiol modification has also been implicated in the dysregulation of alternative heat shock proteins belonging to the HSP90 family (Carbone et al. 2005), thus raising the possibility that such proteins may be particularly susceptible to this form of modification.

While the primary impact of 4HNE covalently binding to heat shock proteins in mature spermatozoa is likely to disrupt chaperoning activity leading to a loss of functionality, this highly reactive aldehyde may have a more pronounced effect in developing germ cells that possess intrinsic proteasomal and lysosomal machinery (Fig. 3). Indeed, insults such as 4HNE exposure are known to accelerate proteolysis through enhancement of cellular proteasome activity in an effort to mitigate the impact of oxidative injury (Carbone et al. 2004; Wang et al. 2012). Paradoxically, however, at higher concentrations, 4HNE can precipitate extensive protein crosslinking thus impairing proteasome activity (Farout et al. 2006; Shringarpure et al. 2000). Nevertheless, alternative lysosomal degradation mechanisms have also been reported for 4HNE-modified proteins (Marques et al. 2004). It is, therefore, feasible that such mechanisms could underpin the loss of susceptible proteins such as HSPA2 from the developing spermatozoa of the patient population. This notion is further supported by the accelerated rate of HSPA2 proteolysis that ensues following ablation of its protective co-chaperone BAG6 in the testes of transgenic *Bag6* null mice (Sasaki et al. 2008).

5 Conclusions

An idiopathic failure of sperm–oocyte recognition ranks among the major reproductive lesions experienced in male infertility patients. Mounting evidence from a number of independent laboratories suggests that this process is commonly impaired because of an underrepresentation of a key heat shock protein, HSPA2, in pathologically defective spermatozoa. Oxidative stress is clearly an

important negative modifier of sperm quality and function, and given that HSPA2 is strongly influenced by this stressor, both during spermatogenesis and post-testicular maturation of the mature gamete, these findings accord with the view that heat shock proteins are critically involved in conferring upon spermatozoa the potential to interact with the oocyte during these sequential phases of sperm maturation (Bromfield and Nixon 2013; Dun et al. 2012). This paradigm suggests that HSPs such as HSPA2 may hold a strategic gate-keeping role, whereby structural perturbations induced by oxidative stress biochemistry lead to the dysfunction of sperm–oocyte recognition. Important new research questions are therefore raised, concerning the incidence of HSPA2 insufficiency in the patient population, the pathways by which this chaperone is incorporated into the differentiating gamete, how such incorporation becomes so dramatically disrupted in cases of infertility, and the mechanisms by which HSPA2 orchestrates the presentation of molecules involved in recognition of the oocyte–cumulus complex. Addressing these questions will have important implications for the diagnosis, treatment, and prevention of infertility, and, in so doing, answer the long-standing call for evidence-based medicine in andrological practice.

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The Potential Functions of Small Heat Shock Proteins in the Uterine Musculature during Pregnancy

Daniel J. MacPhee and E.I. Miskiewicz

Abstract The small heat shock protein B (HSPB) family is comprised of eleven members with many being induced by physiological stressors. In addition to being molecular chaperones, it is clear these proteins also play important roles in cell death regulation, cytoskeletal rearrangements, and immune system activation. These processes are important for the uterine smooth muscle or myometrium during pregnancy as it changes from a quiescent tissue, during the majority of pregnancy, to a powerful and contractile tissue at labor. The initiation and progression of labor within the myometrium also appears to require an inflammatory response as it is infiltrated by immune cells and it produces pro-inflammatory mediators. This chapter summarizes current knowledge on the expression of HSPB family members in the myometrium during pregnancy and speculates on the possible roles of these proteins during myometrial programming and transformation of the myometrium into a possible immune regulatory tissue.

1 The Myometrium during Pregnancy

1.1 Adaptation and Differentiation

The uterine smooth muscle or myometrium undergoes structural, physiological, and biochemical changes throughout pregnancy in order to be transformed into a powerful, contractile tissue for labor and parturition. These changes occur in a series of four intervals during pregnancy and one interval post-partum (Fig. 1).

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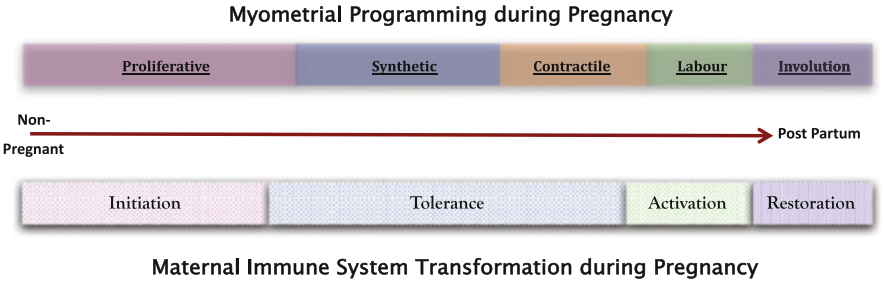

Fig. 1 The myometrium undergoes a program of structural, physiological, and biochemical changes throughout pregnancy. In the proliferation phase, myometrial cells increase in number while in the synthetic phase myometrial cells increase in size under the influence of ever increasing uterine distension caused by a growing fetus(es). The contractile phase is marked by an increased expression of basement membrane matrix proteins while in the labor phase the expression of contraction-associated proteins is elevated. Post-partum involution completes the cycle of myometrial programming as the uterine musculature prepares for the nonpregnant state. In parallel with myometrial programming, the maternal immune system also undergoes transformation with an initial pro-inflammatory initiation phase, an anti-inflammatory tolerance phase, and a second pro-inflammatory activation phase. For detailed information on the phases of myometrial programming and maternal immune system transformation, see Mor and Cardenas (2010) and Shynlova et al. (2013)

Each period is marked by unique characteristics that have been well defined in the pregnant rat model (reviewed by Shynlova et al. 2013). At the beginning of rat pregnancy until day (D) 14, the myometrium enters the proliferation phase. It is marked by increased mRNA expression of insulin-like growth factor-I (IGF-I) and IGF binding protein-1 (IGFBP-1) as well as activation of the mammalian target of rapamycin (mTOR) signaling pathway (Jaffer et al. 2009). Myometrial cells increase in number and the expression of antiapoptotic factors such as B-cell lymphoma 2 (BCL2) is increased within these cells contributing to an overall increase in proliferation (Shynlova et al. 2006). At approximately D14 in the rat, there is clearly a transitional period prior to the synthetic phase. It is marked by increased expression of cleaved poly (ADP-ribose) polymerase (PARP) and activation of an intrinsic apoptotic pathway in the myometrium, yet no indication of significant apoptosis (Shynlova et al. 2009, 2010). It has been proposed that the transition could be a result, in part, of uterine conversion (Reynolds 1949; Shynlova et al. 2009). At this time, the fetus appears to reach a maximal spherical radius resulting in a locally stretched and stressed uterus marked by ischemia (Shynlova et al. 2010). Hypoxia in the rat myometrium was particularly demonstrated in the circular muscle layer at this time. The embryo then undergoes a change to a more ellipsoid shape, presumably releasing local tension stress, alleviating hypoxia, and aiding reestablishment of uterine blood flow. Nonetheless, the embryo(s) continues to grow during pregnancy.

In the subsequent synthetic phase, from D15 to D21, the protein:DNA ratio in the myometrium is increased, cells increase their synthesis of interstitial extracellular matrix proteins (ECM) such as collagen I, and remodel focal adhesions

(MacPhee and Lye 2000; Shynlova et al. 2004). One of the key biophysical stressors during this interval that continues through to the labor phase is increasing uterine distension due to ever increasing fetal growth. The uterus adapts to this distension with increased cellular hypertrophy that contributes to increased uterine weight. For example, before pregnancy the human uterus weighs approximately 40–70 g with a volume of 10 mL, whereas at labor the uterus weighs about 1100–1200 g and can have an average volume of 5 L (Monga and Sanborn 2004). The size of human uterine smooth muscle cells has also been found to increase up to ten times in length and three times in width during gestation (Monga and Sanborn 2004). During late pregnancy in the rat, myocytes undergo a threefold increase in size and the increase is dependent on uterine distension (Shynlova et al. 2010).

In the contractile phase from D21 to D23, there is an increased expression of basement membrane matrix proteins such as fibronectin and increased detection in situ of the associated ITGA5 and ITGB1 integrin receptor subunits in myometrial cells (Shynlova et al. 2004; Williams et al. 2005, 2010). During the final phase of pregnancy, the labor phase at D23, focal adhesion signaling, as well as the expression of contractile-associated proteins (CAPs), such as the gap junction protein GJA1 and oxytocin receptor (OXTR), are elevated (Tabb et al. 1992; Ou et al. 1998; MacPhee and Lye 2000; Li et al. 2007, 2009). In this phase, both endocrine and biophysical pathways are required for induction of CAP gene expression (Ou et al. 1997, 1998; Oldenhof et al. 2002; Li et al. 2007, 2009; Shynlova et al. 2009). Postpartum involution completes the whole cycle of uterine adaptation and differentiation to prepare the uterine musculature for the nonpregnant state. Like wound healing, the process involves the reorganization of the tissue, the production of proteases to assist in this reorganization, the breakdown of extracellular matrix, and cell death. IGF-I, IGFBP-5, and chemokines appear to be important for the involution process (Shynlova et al. 2007, 2009).

1.2 Contributions of Immune System Activation

The rather dated idea that pregnancy is somehow a period of handicapped maternal immunological competence has been rightly challenged (Mor and Cardenas 2010). The immune system can be active and functional during gestation, but most importantly it is tightly regulated. The maternal and fetal–placental immune system work together to achieve this regulation (Mor et al. 2005; Mor 2007; Mor and Cardenas 2010).

For comparison sake and within the context of myometrial adaptation and differentiation, three maternal immunological phases also exist in parallel during pregnancy: an initial pro-inflammatory/initiation phase, an anti-inflammatory/tolerance phase, and a second pro-inflammatory/activation phase that contributes to labor and parturition (Mor and Cardenas 2010; Shynlova et al. 2013; Fig. 1). Focusing on the last pro-inflammatory or activation phase, there is considerable evidence that the beginning and subsequent progression of labor requires an

inflammatory response (Rinaldi et al. 2011). Labor is associated with immune cell infiltration of the myometrium and the increased expression of pro-inflammatory mediators in intrauterine tissues, including the myometrium, such as tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), monocyte chemoattractant protein-1 (MCP-1; also known as C-C chemokine motif ligand 2 or CCL-2), IL-6, and IL-8 (Osmers et al. 1995; Thomson et al. 1999; Young et al. 2002; Kemp et al. 2002; Osman et al. 2003; Esplin et al. 2005; Shynlova et al. 2008; Srikhajon et al. 2014). Uterine stretch appears to be a major player for induction of myometrial production of cytokines/chemokines (Loudon et al. 2004; Shynlova et al. 2008, 2013; Lee et al. 2015). It is possible that stretch-induced production of cytokines/chemokines by the myometrium could increase expression of cell adhesion molecules on uterine blood vessel endothelial cells and stimulate the infiltration of leukocytes into the myometrium. These cells in turn would produce even more cytokines within this tissue aiding cytokine-mediated myometrial contractility.

An important modulator of the pro-inflammatory phase is the transcription factor nuclear factor kappa B (NFkB), which is also an inducer of prostaglandin synthesis via cyclooxygenase-2 (COX-2; Lindstrom and Bennett 2005a, b; Sykes et al. 2014). NFkB expression is induced by pro-inflammatory stimuli such as lipopolysaccharide (LPS), IL-1β, and TNFα and can also promote the synthesis of cytokines demonstrating a feed-forward mechanism of signaling (Belt et al. 1999; Kniss et al. 2001; Lappas et al. 2002). The induction of cytokine expression by NFkB is likely a result of gene promoters for TNFα, IL-1β, IL-8, and IL-6 containing NFkB recognition elements (Lindstrom and Bennett 2005a, b). NFkB may have other important roles as Condon et al. (2006) reported that IL-1β increased binding of NFkB to the inhibitory progesterone receptor promoter potentially reducing uterine quiescence. Confirming the importance of NFkB to the pro-inflammatory process at labor, Khanjani et al. (2011) reported that NFkB was active (i.e., nuclear localization of p65 subunit) in both the upper and lower human uterine segments before labor onset. Furthermore, when p65 NFkB was overexpressed in human uterine myocytes, it led to phosphorylation and nuclear localization of p65 and p50 subunits and the increased expression of 38 immunity and inflammation-related genes.

2 The HSPB Family

The discovery of the heat shock response over 50 years ago by Dr. Ferrucio Ritossa is just another example of how curiosity and basic research can initiate the building of a knowledge foundation that subsequently results in much larger and impactful future scientific advancements. Dr. Ritossa was researching nucleic acid synthesis in Drosophila salivary gland puffs and reported increased transcriptional activity when the gland cells were inadvertently exposed to higher than normal temperature (Ritossa 1962, 1996; De Maio 2011; De Maio et al. 2012). Heat shock proteins were subsequently discovered 12 years later (Tissieres et al. 1974). The heat shock or stress response is now known to occur in response to many stressors and the

identification of families of stress proteins, co-chaperones, and their importance in diverse organisms in all kingdoms (e.g., from archea to higher eukaryotes) as well as disease are internationally recognized (Kampinga et al. 2009; Kampinga and Garrido 2012; Garrido et al. 2012; Morrow and Tanguay 2012).

The small heat shock protein B (HSPB) family is comprised of eleven small molecular weight proteins (15–40 kDa; Table 1) that are key for cellular homeostasis and are induced by physiological stressors such as temperature, oxidative stress, and biophysical forces (reviewed by Laskowksa et al. 2010; Kampinga and Garrido 2012). Canonically, HSPB members act as ATP-independent molecular chaperones that bind to unfolded proteins following stress and prevent them from irreversible aggregation until they are passed on to the ATP-dependent chaperone networks (e.g., HSP70/HSPA family) for refolding (Laskowska et al. 2010). However, they also assist in other processes such as cell death regulation, cytoskeletal rearrangements, and immune system activation (Acunzo et al. 2012; van Noort et al. 2012; Wettstein et al. 2012). Importantly, many of the HSPB members can heteroligomerize with one another, adding greater functional complexity (Zantema et al. 1992; Fontaine et al. 2005).

The domain organization of the HSPB family members has been recently reviewed (Haslbeck et al. 2015). Briefly, members are characterized by a signature

Table 1 Nomenclature and tissue distribution of the human HSPB family of heat shock proteins

Gene name[a]	Protein name	Common older names	Predicted molecular weight (kDa)	Main tissue distribution	Heat stress inducible (Yes/No)
HSPB1	HSPB1	HSP27	22.8	Ubiquitous	Y
HSPB2	HSPB2	MKBP	20.2	Muscle	N
HSPB3	HSPB3	HSPL27	17.0	Muscle	N
CRYAA	CRYAA	αA-crystallin, HSPB4	19.9	Lens	Y
CRYAB	CRYAB	αB-crystallin, HSPB5	20.2	Ubiquitous	Y
HSPB6	HSPB6	HSP20	17.1	Ubiquitous	Y
HSPB7	HSPB7	cvHSP	18.6	Muscle	N
HSPB8	HSPB8	HSP22	21.6	Ubiquitous	Y
HSPB9	HSPB9	CT51	17.5	Testis	Unknown
HSPB10	HSPB10	ODF1	28.4	Testis	Unknown
HSPB11	HSPB11	IFT25	16.2	Testis, Placenta	Unknown

Information from Hu et al. (2007), Kampinga et al. (2009), Garrido et al. (2012), Kampinga et al. (2015), UNIPROT; www.uniprot.org

Abbreviations: *HSP27* heat shock protein 27 kDa, *MKBP* myotonic dystrophy protein kinase binding protein, *HSPL27* heat shock protein like 27 kDa, *HSP20* heat shock protein 20 kDa, *cvHSP* cardiovascular heat shock protein, *HSP22* heat shock protein 22 kDa, *CT51* cancer/testis antigen 51, *ODF1* outer dense fiber of sperm tails 1, *IFT* intraflagellar transport protein homology

[a]Gene name is provided based on the HUGO Gene Nomenclature Committee, www.genenames.org

conserved C-terminal region named the α-crystallin domain, a more variable N-terminal sequence, and in most cases a short variable C-terminal region. Although the α-crystallin domain has a low degree of amino acid conservation, the length and structure is well conserved (Kriehuber et al. 2010). Structurally, the domain produces a compact β-sheet, and it appears that the structure is important for dimerization, although it is not sufficient for oligomerization alone (Bagneris et al. 2009; Laganowsky et al. 2010; Baranova et al. 2011; Clark et al. 2011). The C-terminal region in the HSPB family differs considerably between members (Kriehuber et al. 2010; Chen et al. 2010; Basha et al. 2012; Delbecq and Klevit 2013), while the N-terminal region appears to be very flexible and differs substantially in length and amino acid composition. For example, HSPB1 contains a WDPF motif in this domain, which is required for oligomer formation. Overall though, amino acid residues in all three regions of HSPB members are needed for oligomerization (Delbecq and Klevit 2013).

Among posttranslational modifications of the HSPB family, such as thiolation or glyoxylation, phosphorylation of these proteins on serine residues is especially critical for regulation of structure and function (Mymrikov et al. 2011). In fact, it is known to be one of the earliest events induced by a stress like heat shock, oxidative stress, and stimuli such as cytokines and growth factors (Landry et al. 1992). For example, HSPB1 phosphorylation results in dissociation of large oligomers of HSPB1 and loss of chaperoning activity (Kato et al. 1994).

3 Uterine Expression of HSPB Members

3.1 HSPB1

HSPB1 was examined in human endometrial stromal cells during in vivo and in vitro decidualization (Tabanelli et al. 1992; Tang et al. 1993; Shah et al. 1998). In addition, an HSPB-like protein was detected in mouse uterine endometrial stromal cells undergoing decidualization (Bany and Schultz 2001). Ciocca et al. (1996) also detected HSPB1 in situ within rat uterine decidual cells during pregnancy and pseudopregnancy, but only briefly indicated expression in the myometrium. Thus, despite these reports, for quite some time there was a complete lack of information regarding the spatiotemporal expression of HSPB1 in the myometrium during late pregnancy and labor.

White et al. (2005) then demonstrated that *Hspb1* mRNA expression was significantly increased during late pregnancy in the rat myometrium. Specifically, *Hspb1* mRNA expression became significantly elevated between D17 and D22 of pregnancy, inclusive, compared to nonpregnant (NP), D6, D12, D23 (labor), and post-partum (PP) time points (White et al. 2005). Immunoblot analysis utilizing antisera that detected both non-phosphorylated and phosphorylated HSPB1 (total HSPB1) demonstrated that HSPB1 levels increased significantly during late

pregnancy, labor, and PP. Furthermore, Serine (Ser)-15 phosphorylated HSPB1 (pSer15-HSPB1) detection significantly increased during late pregnancy and labor (synthetic, contractile and labor phases) and paralleled the increased detection of total HSPB1.

Two phosphorylation sites were reported for HSPB1 in rodents, Ser-15 and Ser-86 (homologous to Ser-82 in humans and Ser-90 in hamsters; Gaestel et al. 1991). The latter site is necessary for the dissociation of large multimers, but at the cellular level it is not always sufficient (Lambert et al. 1999; Gaestel 2002). Ser-15 phosphorylation of HSPB1 may produce a conformational change in the protein that aids the direct binding of HSPB1 with actin microfilaments (Lambert et al. 1999). A number of past reports have indicated a role for HSPB1 in actin polymerization, remodeling, and even cross-bridge cycling in smooth muscle cells (Miron et al. 1991; Lavoie et al. 1993a, b; Benndorf et al. 1994; Ibitayo et al. 1999). Work by Bitar (2002) demonstrated that agonist-induced HSPB1 phosphorylation promoted actin–myosin association likely through interaction with tropomyosin. Therefore, HSPB1 phosphorylation could be necessary for contraction of smooth muscle.

In rat myometrium, immunocytochemical analysis of pSer15-HSPB1 expression in situ demonstrated that in circular muscle it became detectable in peri-nuclear and membrane regions on D19-D22, but was primarily restricted to the cytoplasm on D23-PP (White et al. 2005). In contrast, pSer15-HSPB1 in longitudinal muscle was primarily detected in myocyte membranes on D15-D22 and then also became detectable in the cytoplasm of myocytes on D23 and PP. All of these results and data from others indicating a role for pSer15-HSPB1 in actin–myosin interaction led to our hypothesis that HSPB1 was likely a contraction-associated protein, although more definitive proof for such a role is presently a significant knowledge gap. A role for pSer15-HSPB1 as a chaperone to protect the actin cytoskeleton also still cannot be ruled out at this time (Liang and MacRae 1997; Mounier and Arrigo 2002).

Soon thereafter, MacIntyre et al. (2008) examined HSPB1 expression in human myometrium. They used immunoblot analysis to report a significant threefold increase in expression of pSer15-HSPB1 in laboring human myometrium compared to non-laboring myometrium. HSPB1 and smooth muscle actin also co-immunoprecipitated from both non-laboring and laboring myometrial lysates, indicating a specific association of the two proteins. This was also supported by immunofluorescence analysis showing the presence of HSPB1 in laboring myometrium in cytoplasmic regions, but in fibrillary arrangements where it co-localized with smooth muscle actin. The authors also provided additional support that pSer15-HSPB1 expression was associated with contraction by treating non-laboring human myometrial strips, obtained from the upper regions of the lower uterine segment, with oxytocin and the resulting significant increase in contractility matched by a significant increase in pSer15-HSPB1 expression.

Despite knowledge of HSPB1 expression in rat and human myometrium, it was not known how expression was regulated in this tissue. HSPB1 expression was reportedly induced by stretch of epithelial cells and heavy resistance training of

skeletal muscle (Murlasits et al. 2006; Luo et al. 2007). Chaudhuri and Smith (2008) also reported that cyclic mechanical stress of airway smooth muscle cells resulted in increased HSPB1 phosphorylation. Interestingly, our report of significantly induced HSPB1 expression during late pregnancy and labor (White et al. 2005) paralleled the significant increase in mechanical stress or distension exerted on the myometrium due to growing fetuses, yet the role of uterine distension on HSPB1 expression within the uterine musculature during pregnancy was undetermined at that time.

Utilizing a unilaterally pregnant rat model, we examined the effect of uterine distension on myometrial HSPB1 expression (White and MacPhee 2011). *Hspb1* mRNA and pSer15-HSPB1 protein expression were significantly elevated in distended gravid uterine horns at both D19 and D23 of gestation compared to nongravid horns. Similarly, pSer15-HSPB1 protein detection in situ was only observed in the distended horns compared to the nongravid horns at both time points. *Hspb1* mRNA and pSer15-HSPB1 protein expression were also markedly increased in ovariectomized, nonpregnant rat myometrium distended for 24 h with laminaria tents compared to empty horns. This ruled out the necessity, but not necessarily the ability, of ovarian hormones to contribute to the increased expression observed during pregnancy. Therefore, uterine distension during the synthetic, contractile, and labor phases does play a major role in the stimulation of myometrial HSPB1 expression. We hypothesized that this response could regulate actin cytoskeleton dynamics at focal adhesion sites and support uterine distension-induced hypertrophy and subsequent focal adhesion reorganization during late pregnancy (White and MacPhee 2011). Evidence to support this possibility can be found in reports such as During et al. (2007) who demonstrated that HSPB1 was a G-actin sequestering protein and that HSPB1 phosphorylation enhanced actin filament assembly. The promotion of actin formation and actin–myosin interaction by HSPB1 were shown to be essential for the contraction of colonic smooth muscle (Bitar 2002), and it was hypothesized that the mechanism involved modulation of caldesmon association with tropomyosin mediated by phosphorylated HSPB1 (Bitar 2002; Somara and Bitar 2006). Taken with the findings of stretch inducing phosphorylation of caldesmon in the myometrium (Li et al. 2009), a specific association or relationship of HSPB1, caldesmon and stretch in uterine smooth muscle contraction may exist at labor. Further identification of the exact mechanism(s) of action of HSPB1 on the myometrial cell actin cytoskeleton and on contraction is urgently needed, but will require a molecular approach at the cellular level.

3.2 CRYAB (HSPB5)

CRYAB or HSPB5, previously known as αB-crystallin, was discovered as a highly abundant eye lens protein that helped maintain the transparency of the structure (Bloemendal 1982; Clark et al. 2012). CRYAB maintains lens transparency by acting as a molecular chaperone to aid cytoprotection and prevent aggregation of

denatured proteins (Horwitz 1992). CRYAB is expressed in many tissues, and mutations in CRYAB can lead to congenital cataracts, cardiac myopathies, and neurodegenerative diseases (Acunzo et al. 2012; Boncoraglio et al. 2012). Of note, CRYAB phosphorylation on Ser-59 residues also regulates CRYAB–actin interaction (Singh et al. 2007).

From an immune system perspective, CRYAB was shown to induce an IL-10 regulatory macrophage immune response at low concentrations, while at higher concentrations it increased T-cell production of interferon-γ (IFN-γ), which activated macrophages (van Noort et al. 2010, 2012). CRYAB also altered the immune system by increasing the expression of endothelial cell adhesion molecules, such as intercellular adhesion molecule-1 and E-selectin, that are responsible for slowing leukocyte rolling and aiding leukocyte entry into tissues (Dieterich et al. 2013). The interaction of CRYAB with actin and its role in immune modulation subsequently made examination of this protein in the myometrium during pregnancy very important.

MacIntyre et al. (2008) first examined CRYAB expression in non-laboring and laboring human myometrial tissue. Using a combination of two-dimensional-difference gel electrophoresis and immunoblot analysis, they reported a 69–71% reduction in CRYAB protein abundance in human laboring myometrium compared to non-laboring. Importantly, the authors demonstrated through immunoprecipitation assays that HSPB1 and CRYAB were specifically associated with one another in both non-laboring and laboring myometrium. Zantema et al. (1992) had earlier indicated that HSPB1 and CRYAB could complex together.

We have recently examined expression and phosphorylation of CRYAB in rat myometrium during pregnancy and labor (Nicoletti et al. 2016). Myometrial CRYAB protein expression significantly increased from D15 through to D23 (synthetic—labor phases). In correlation with these findings, pSer59-CRYAB protein levels also significantly increased during this period. Furthermore, both total and pSer59-CRYAB were detected in the cytoplasm of myocytes within both uterine muscle layers mid- to late pregnancy and at labor. These results are in contrast to MacIntyre et al. (2008) above, but this may reflect species-specific differences in total CRYAB expression and localization in the myometrium at labor or be related to the collection of myometrium from the lower uterine segment. We also discovered that uterine stretch was a major player in regulation of CRYAB expression. Both total and pSer59-CRYAB protein expression were significantly elevated in gravid uterine horns at both D19 and D23 of gestation compared to contralateral nongravid horns of unilaterally pregnant rats (Nicoletti et al. 2016).

To better understand the potential pSer59-CRYAB interactome within the myometrium, Nicoletti et al. (2016) also examined the spatial localization of CRYAB with potential partners using the hTERT—human myometrium cell line (Condon et al. 2002). pSer59-CRYAB co-localized with the focal adhesion protein FERMT2 at the ends of actin filaments. pSer59-CRYAB also co-localized with the exosomal marker CD63 within vesicle-like structures. Focal adhesions or smooth muscle dense plaques are sites on the plasma membrane where integrin receptors cluster and integrin activators such as FERMT2 help initiate a structural link

between the ECM and the actin cytoskeleton. Actin filaments are very active at focal adhesion locations, remodeling, and anchoring the plasma membrane to the ECM and to other cells, and focal adhesion signaling is important for promoting myometrial cell contraction in late pregnancy (Launay et al. 2006; Li et al. 2007). Due to the increase in pSer59-CRYAB expression during late pregnancy and labor and induction by uterine distension, we speculate that pSer59-CRYAB may be part of a mechano-adaptive response, perhaps in partnership with pSer15-HSPB1, to modulate actin polymerization dynamics at focal adhesions in the myometrium during late pregnancy and to facilitate subsequent phasic labor contractions.

In contrast to a role with the cytoskeleton, HSPB proteins have been shown to be extracellular signaling molecules that can be secreted via exosomes (Clayton et al. 2005; Rayner et al. 2008; Sreekumar et al. 2010; Gangalum et al. 2011; reviewed by van Noort et al. 2012). Since pSer59-CRYAB readily co-immunolocalized with the exosome marker CD63 (Nicoletti et al. 2016), this suggests that pSer59-CRYAB may be localized in vesicles destined to be released as exosomes from myometrial cells. However, exosomal vesicles have an endocytic origin (Thery et al. 2002), and late endosomes can also contain CD63. Thus, we cannot rule out localization of pSer59-CRYAB to these late endosomes in myometrial cells. Exosome production was demonstrated in vascular smooth muscle and hypothesized to occur in myometrium (Liao et al. 2000; Martin-Ventura et al. 2004; Cretoiu et al. 2013). The uptake of exosomes by macrophages through phagocytosis and the subsequent release of contents were also confirmed (Feng et al. 2010). In this manner, CRYAB and other HSPB family members can signal to macrophages or macrophage-like cells and induce innate immune responses (Bhat and Sharma 1999; van Noort et al. 2010). Interestingly, van Noort et al. (2010) demonstrated that HSPB family members promote activation of macrophages into an immune regulatory state that stimulates tissue repair and attenuates inflammation. We speculate that the detection of CRYAB in myometrium post-partum may be indicative of such a role in tissue repair and signaling to immune cells at this time since the involution process is similar to wound healing (Shynlova et al. 2013; Nicoletti et al. 2016). The immune response, however, likely depends on the local concentration of CRYAB as high local concentrations can stimulate a pro-inflammatory immune response (van Noort et al. 2010, 2012). As stated earlier (see Sect. 1.2), the maternal immune system during pregnancy and labor goes through an immunological transformation in concert with myometrial programming (Shynlova et al. 2013). Thus, CRYAB may have chronologically specific anti-inflammatory and pro-inflammatory roles in the myometrium during pregnancy and post-partum to aid this immunological transformation.

3.3 HSPB8 and BAG3

HSPB8 is highly expressed in smooth muscle, and it is an important provider of protein quality control such as in protecting cells from the accumulation of

aggregated proteins including other HSPs and their targets (Mymrikov et al. 2011). HSPB8 was also found to interact with the adapter protein Bcl2-associated athanogene 3 (BAG3) (Carra et al. 2008; Fuchs et al. 2010; Hishiya et al. 2011). The BAG family of co-chaperones is capable of interacting with the ATPase domain of heat shock cognate 70 protein (HSPA8) through their structural BAG domain (Takayama et al. 1999; Rosati et al. 2007). Like some sHSPs, *bag3* gene expression is stress inducible (Rosati et al. 2011). BAG3 is also known to serve as a stimulator of a protein quality control pathway known as macroautophagy. This is a bulk degradation process in which cells collect portions of the cytoplasm, organelles, and aggregated proteins into vesicles known as autophagosomes for transport to lysosomes for degradation (Ravikumar et al. 2009). When HSPB8 interacts with BAG3, it can be part of a multi-heteromeric complex that includes additional proteins such as HSPA8 and the ubiquitin ligase Strip1 homology and U-box containing translation protein 1 (STUB1; also known as CHIP) to aid autophagy (Arndt et al. 2010). Interestingly, adenoviral-mediated overexpression of HSPB8 in rat cardiac myocytes was shown to be associated with a 37% increase in the protein/DNA ratio, suggesting a role in stimulating hypertrophy (Depre et al. 2002). This led us to investigate HSPB8 expression in the myometrium during pregnancy.

Both HSPB8 and BAG3 protein expression were significantly elevated during the synthetic phase of myometrial differentiation marked by initiation of uterine distension and myometrial hypertrophy (Marsh et al. 2015). Furthermore, HSPB8 and BAG3 were predominantly localized to myometrial cells throughout pregnancy with intense cytoplasmic HSPB8 and BAG3 detection on D15 and D17 in both longitudinal and circular muscle layers. Carra et al. (2008) had suggested that a chaperone complex comprised of HSPB8 and BAG3 proteins could be formed. Using immunoprecipitation assays, we demonstrated that HSPB8 and BAG3 formed a signaling complex in vivo in pregnant rat myometrium (Marsh et al. 2015). Analysis of HSPB8 and BAG3 protein expression in myometrium from unilateral pregnancies also revealed that expression of both proteins was significantly increased on D15 of gestation by uterine distension. Since muscle tissue relies on an effective proteostasis network (Arndt et al. 2010), we postulate that HSPB8 and BAG3 are important to help maintain protein homeostasis, particularly during the early synthetic phase of myometrial differentiation where they could prevent aggregation or promote degradation of misfolded proteins during initiation of myometrial hypertrophy. The identity of specific HSPB8 and/or BAG3 targets and the necessity of these proteins for myometrial cell viability and function must be a priority to further understand the role(s) of these chaperones during pregnancy.

3.4 HSPB6

HSPB6 (formerly HSP20) is expressed in practically all tissues, but reaches a maximal level of 1.3% of total proteins in skeletal and smooth muscle (Mymrikov et al. 2011). This 20 kDa protein can be phosphorylated by both cAMP- and

cGMP-dependent protein kinase (PKA/PKG), and increases in phosphorylation were associated with relaxation of smooth muscle (Beall et al. 1999). Beall et al. (1999) also showed that the introduction of phosphopeptide analogs of HSPB6 into bovine carotid artery smooth muscle inhibited agonist-induced muscle contractions. Overall, the importance of HSPB6 in smooth muscle relaxation made it a priority to investigate it in the myometrium during pregnancy.

We demonstrated that HSPB6 mRNA and total HSPB6 protein in rat myometrium were highly expressed during early and midpregnancy (proliferative and synthetic phases) and then expression decreased during late pregnancy and labor (Cross et al. 2007). Strikingly, the detection of HSPB6 in plasma membrane-associated regions in situ decreased after D15 of rat pregnancy in both circular and longitudinal muscle layers. Tyson et al. (2008) then examined HSPB6 expression in non-laboring and laboring human myometrium and provided evidence that it could promote myometrial relaxation. Using a combination of SDS-PAGE, 2D–SDS-PAGE, and immunoblot analysis, they showed that total and phosphorylated HSPB6 (no specific phosphorylation site was identified) levels were unchanged in term non-laboring and laboring myometrium. Furthermore, HSPB1 and HSPB6 were found to be associated with one another in term pregnant myometrial lysates using immunoprecipitation assays. Functionally, the researchers showed that phosphorylated HSPB6 could have a novel role in regulating cyclic nucleotide-mediated relaxation of myometrium. Using term pregnant human myometrial strips, the authors exposed the tissue to forskolin or rolipram (a phosphodiesterase inhibitor), which resulted in relaxation of the spontaneously contracting strips and also a significant increase in phosphorylated HSPB6 levels in the tissue extracts. The latter result was not observed when the strips were relaxed with the calcium channel blocker nifedipine.

The work by Tyson et al. (2008) did not identify the expression of the specific phosphorylated form of HSPB6, and this protein can undergo multisite phosphorylation (Mymrikov et al. 2011). Thus, we examined the expression of pSer16-HSPB6 in rat myometrium throughout pregnancy since this site was known to be phosphorylated by PKA and associated with HSPB6 promotion of smooth muscle relaxation (Beall et al. 1999). We found that pSer16-HSPB6 expression was detectable throughout pregnancy and labor, but it was significantly elevated at D19, D21, and especially D22 of pregnancy (Fig. 2). Thus, it was present to potentially promote myometrial relaxation during pregnancy and during phasic labor contractions.

Upon phosphorylation, HSPB6 had been shown to associate with actin (Rembold et al. 2000) and the actin-binding protein α-actinin (Tessier et al. 2003), which is located at focal adhesions in smooth muscle cells. Amino acid residues 110–121 of HSPB6 bear sequence homology with the actin binding region of both cardiac and skeletal troponin I, and this region appears necessary for binding of HSPB6 to actin filaments (Rembold et al. 2000). This binding may inhibit cross-bridge cycling (myosin–actin interactions), thus leading to relaxation of smooth muscle (Rembold et al. 2001). However, this model of HSPB6 being a genuine actin-binding protein has been called into question (Mymrikov et al. 2011).

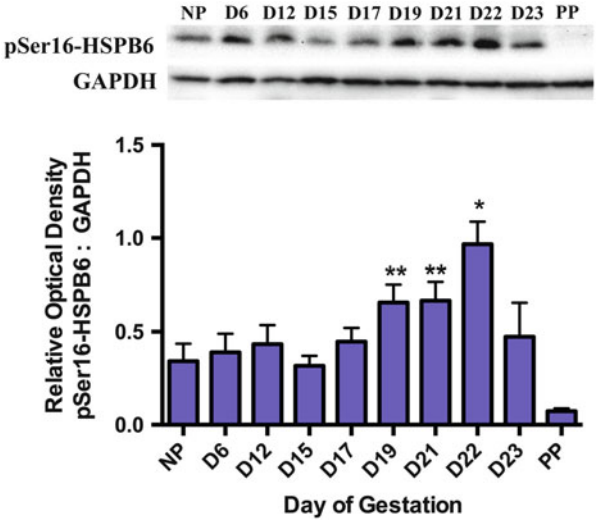

Fig. 2 Expression of pSer16-HSPB6 is highly induced during late pregnancy in the rat myometrium. Representative immunoblots and densitometric analysis of pSer16-HSPB6 and GAPDH expression during gestation are shown. pSer16-HSPB6 protein expression was significantly increased at D22 compared to nonpregnant (NP), D6-D17, and post-partum (PP) time points ($*P < 0.05$). pSer16-HSPB6 protein expression was also significantly increased at D19 and D21 compared to PP ($**P < 0.05$). Data presented are from four independent experiments ($n = 4$), and error bars represent the standard error of the mean. pSer16-HSPB6 was detected with polyclonal rabbit antisera specific to the phosphorylated form of the protein (Catalog # ab58522, Abcam, Cambridge, MA, US). GAPDH was also detected with rabbit polyclonal antisera (Catalog # ab9485, Abcam). pSer16-HSPB6, serine-16 phosphorylated HSPB6, GAPDH, glyceraldehyde 3-phosphate dehydrogenase

Another possibility stems from findings that phosphorylated HSPB6 can bind the adapter protein 14–3-3, potentially displacing phosphorylated cofilin. Cofilin would then be free to be dephosphorylated and to promote actin depolymerization (Birkenfeld et al. 2003; Dreiza et al. 2005; Komalavilas et al. 2008; Mymrikov et al. 2011).

Recently, a role for acetylation of HSPB6 in myometrial relaxation was uncovered (Karolczak-Bayatti et al. 2011). The authors utilized an antisera specific for HSPB6 acetylated on lysine 160 (Ac-HSPB6) and showed by immunoblot analysis that Ac-HSPB6 was highly expressed in pregnant human myometrial tissue compared to nonpregnant myometrium. They also reported that Ac-HSPB6 complexed with histone deacetylase-8 (HDAC-8) and cofilin in myometrial tissue and that Ac-HSPB6, independent of HSPB6 phosphorylation, increased levels of dephosphorylated cofilin. Thus, it seems likely that either pSer16-HSPB6 and/or Ac-HSPB6 could be involved in a pathway(s) resulting in displacement of 14–3-3 from phosphorylated cofilin, the dephosphorylation of cofilin, and subsequent modulation of actin filament dynamics leading to relaxation. Since pSer15-HSPB1 was highly expressed at labor and controlled actin filament assembly

(White et al. 2005; During et al. 2007), pSer15-HSPB1 could partner with pSer16-HSPB6 or Ac-HSPB6 at labor to form the dynamic signaling complex required for phasic uterine contractions. It would seem prudent that the use of nonnuclear histone acetyltransferases, HDAC8 inhibitors, PKA activators, or phosphopeptide analogs of HSPB6 should be explored as potential tocolytics to address preterm labor.

4 Future Directions

As described in Sect. 3.2, HSPB proteins were shown to be extracellular signaling molecules that can be secreted via exosomes (Clayton et al. 2005; Rayner et al. 2008; Sreekumar et al. 2010; Gangalum et al. 2011; reviewed by van Noort et al. 2012). Since we recently detected CRYAB in vesicle-like structures within hTERT-human myometrial cells that also contained the exosome marker CD63 (Nicoletti et al. 2016), it is likely that myometrial cells also secrete HSPB-containing exosomes. Thus, it is a priority to demonstrate more definitively the production of these vesicles and their various cargos from myometrial cells as vehicles for intercellular communication during pregnancy and labor.

A direct consequence of this type of intercellular communication could be a role in modulating maternal immune activation in intrauterine tissues, including the myometrium. This would help link more categorically the myometrial differentiation pathway with immune activation in the uterus. In fact, Shynlova et al. (2013) have indicated that the myometrium can be an immune regulatory tissue and regulate how groups of immune cells contribute to labor and subsequent involution. Many HSPB family members, including HSPB1 and HSPB5, can induce the production of cytokines/chemokines in various tissues or cells (van Noort et al. 2010, 2012). We speculate that stretch-induced HSPB expression plays a role in inducing and regulating cytokine/chemokine expression in the myometrium, perhaps through NFkB, and investigation of these possible roles is a priority.

Despite broad evidence in the research field that HSPB protein members can influence the actin cytoskeleton and perhaps even associate with actin filaments, clear confirmation of these properties in myometrial cells is absent. Recently, Clarke and Mearow (2013) directly demonstrated an interaction between HSPB1 and F-actin in rat pheochromocytoma PC12 cells. A similar strategy is urgently required for HSPB members in myometrial cells including any direct role in contraction. Elucidation of such mechanism(s) of action will solidify the importance of these HSPB proteins for pregnancy and parturition.

In summary, HSPB family proteins appear to have roles in myometrial relaxation, contraction, and proteostasis during pregnancy and labor (Fig. 3). Future work will hopefully establish the exact mechanism(s) of their action, but it would have been hard to imagine that the discovery of the heat shock/stress response in fruit fly salivary glands so many years ago would have led to investigation of HSPB proteins

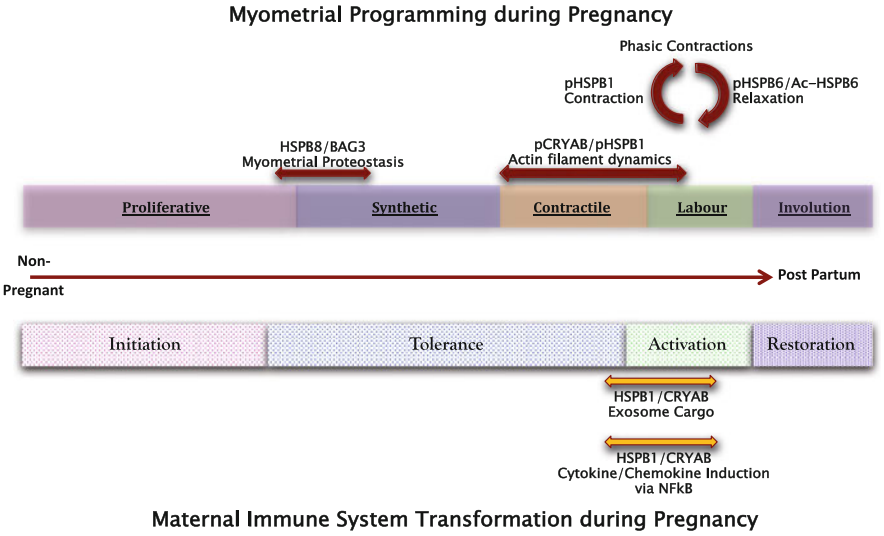

Fig. 3 Potential functions for HSPB family proteins in myometrial programming and maternal immune system transformation. With respect to myometrial programming, muscle tissue relies on an effective proteostasis network; thus, we postulate that HSPB8 and the co-chaperone BAG3 are important to help maintain protein homeostasis, particularly during the early synthetic phase of myometrial differentiation where they could prevent aggregation or promote degradation of misfolded proteins during initiation of hypertrophy. We also speculate that pSer59-CRYAB may be part of a mechano-adaptive response, perhaps in partnership with pSer15-HSPB1, to modulate actin polymerization dynamics at focal adhesions in the myometrium during late pregnancy and thus facilitate subsequent labor contractions. pSer15-HSPB1 could also partner with pSer16-HSPB6 or Ac-HSPB6 to form the dynamic signaling complex required for such phasic contractions. HSPB proteins have been shown to be extracellular signaling molecules that can be secreted via exosomes, and it is possible that HSPB1 and CRYAB could be cargo for such structures. A direct consequence of this type of intercellular communication could be a role in modulating maternal immune activation in intrauterine tissues, including the myometrium**.** It is possible that HSPB expression during late pregnancy and labor plays a role in inducing and regulating cytokine/chemokine expression in the myometrium, perhaps through NFkB

in mammalian reproduction. We have to thank Dr. Ritossa for his observations and demonstration that for scientific advancement, curiosity is where it all begins.

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The Role of Hsp70 in the Regulation of Autophagy in Gametogenesis, Pregnancy, and Parturition

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Abstract Induction of the 70 kDa heat shock protein (hsp70) and autophagy are two major mechanisms that promote cell homeostasis during the rapid cell growth and differentiation characteristic of reproduction. Hsp70 insures proper assembly, conformation, and intracellular transport of nascent proteins. Autophagy removes from the cytoplasm proteins, other macromolecules, and organelles that are no longer functional or needed and recycles their components for synthesis of new products under nutritionally limiting conditions. Hsp70 inhibits autophagy and so a proper balance between these two processes is essential for optimal germ cell production and survival and pregnancy progression. A marked inhibition in autophagy and a concomitant increase in hsp70 at term is a trigger for parturition. Excessive external or endogenous stress that induces a high level of hsp70 production can lead to a non-physiological inhibition of autophagy, resulting in altered spermatogenesis, premature ovarian failure, and complications of pregnancy including preeclampsia, intrauterine growth restriction, and preterm birth.

1 Introduction

The inducible 70 kDa heat shock protein (hsp70), also known as HSPA1A, is a major component of the stress response. When a cell encounters conditions that alter its physiological milieu—elevated temperature, inflammation, infection, toxic chemicals, oxidative stress, hypoxia, rapid growth, differentiation—synthesis of hsp70 is greatly upregulated. Hsp70 binds to nascent proteins and either prevents their denaturation, assists in maintaining their proper three-dimensional conformation, facilitates intracellular transport, or marks irreversibly degraded proteins for exclusion from the cell (Parcell and Lindquist 1993). A second major response to stress is the induction of autophagy. Unlike hsp70 whose major function is to preserve protein function, the major stress-related function of autophagy is to

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remove altered or dysfunctional components from the cytoplasm. A double-membraned structure called an autophagosome is formed and surrounds the component to be degraded—either a denatured or aggregated protein, other macromolecules that are no longer needed, aged or dysfunctional organelles such as mitochondria and inflammasomes, intracellular viruses or bacteria, or microbial components or products. The autophagosome then fuses with a lysosome and the captured components are degraded by lysosomal enzymes. The resulting amino acid, nucleic acid, lipid and carbohydrate constituents are returned to the cytoplasm for reutilization (Levine 2005). Autophagy can thus be seen as a major mechanism to promote cell survival in periods of nutrient limitation.

Under most, but not all circumstances, hsp70 induction and autophagy are opposing responses with hsp70 being dominant. The induction of hsp70 was shown to result in inhibition of autophagy both in vitro (Dokladny et al. 2013) and in vivo (Dokladny et al. 2015). Sera from pregnant women induced hsp70 in peripheral blood mononuclear cells, and the intracellular concentration of hsp70 was strongly inversely related to the extent of autophagy in these cells (Kanninen et al. 2013a, b, 2016). The major inhibitor of autophagy induction is an intracellular complex known as mammalian target of rapamycin complex 1 (mTORC1) (Zoncu et al. 2011). Induction of hsp70 results in the phosphorylation and activation of mTORC1. In addition, activated mTORC1 phosphorylates and activates heat shock factor 1, the transcription factor that induces transcription of the gene coding for hsp70 (Chou et al. 2012).

The present review will highlight what is known about the roles of autophagy and hsp70 in gametogenesis, pregnancy and parturition and how perturbations in their relative rates of production and interactions contribute to infertility and disturbances of pregnancy.

2 Gametogenesis

In most male mammals, the testes descend before birth into a scrotal sac located outside of the body cavity. In men the scrotal temperature is 2–4 °C lower than the internal body temperature. This decreased temperature promotes optimal spermatogenesis that initiates at age 10–11. If the testes do not descend spermatogenesis is impaired (Niedzielski et al. 2016). Although it is most probable that heat shock protein synthesis is upregulated in testes subjected to elevated temperature, this has been poorly studied. The presence of a varicocele, an enlargement of veins in the testicles leading to impaired spermatogenesis and an increased testicular temperature, has been shown to result in increased expression of hsp70 (Chan et al. 2013). Similarly, a requirement for autophagy in spermatogenesis has not been fully investigated. In the most extensive study performed in mice, it was shown that a subunit of protein phosphatase 1 promotes autophagy during spermatogenesis and that inhibition of autophagy leads to impaired sperm production (Zhang et al. 2016). Although yet to be demonstrated, it is likely that the induction of hsp70 and

possibly other heat shock proteins in the testicles as a consequence of exposure to elevated temperature, infection, oxidative stress or inflammation following testicular trauma inhibits autophagy and, therefore, impairs spermatogenesis.

Unlike spermatogenesis that is initiated during early adolescence, the development of ovarian follicles occurs and is completed prior to birth. These follicles must remain viable until the woman reaches sexual maturity, and even then, only one follicle is released during a normal menstrual cycle. Autophagy appears to be involved both in maintaining the viability of oocytes during embryogenesis (Gawriluk et al. 2011) and in the interval between birth and puberty (Reddy et al. 2008). In mice when a gene coding for a protein, tuberous sclerosis complex 1, that inhibits mTORC1 activity is knocked out, autophagy is inhibited and ovarian follicles become prematurely activated (Adhikari et al. 2010). Recent evidence from a study in mice has demonstrated that the induction of autophagy by interleukin-33 is a major mechanism to maintain the function and viability of ovarian tissue by the prompt removal of unneeded debris (Wu et al. 2015a, b). While direct evidence is sparse (Sirotkin and Bauer 2011), it is likely that hsp70 induction in the ovary as a consequence of infection, sterile inflammation, oxidative stress, or exposure to an environmental toxin downregulates autophagy and thereby increases susceptibility to premature ovarian failure.

3 Embryogenesis

Heat shock proteins are among the first products that are produced following initiation of gene transcription in the fertilized oocyte. The stress-induced induction of hsp70 has been identified as early as the blastocyst stage (Wittig et al. 1983; Christians et al. 1997). The development of mouse embryos in vitro is inhibited when monoclonal antibodies to hsp60 or hsp70 are included in the culture medium (Neuer et al. 1998). Thus, it is likely that the stresses associated with early embryo development and differentiation result in the induction of hsp70 to facilitate optimal protein activity. Amazingly, autophagy has been identified as early as four hours after sperm penetration of the oocyte. It is believed that this is necessary to remove any sperm mitochondria that might be inside the fertilized egg as well as to sequester oocyte macromolecules that are no longer needed and that whose presence may impede development (Sato and Sato 2011, 2012). Mouse embryos whose ability to induce autophagy has been genetically impaired die at the 4–8 cell stage (Tsukamoto et al. 2008). Autophagy induction in pregnancy has been reported to promote the proliferation and activity of regulatory T lymphocytes (Treg cells) that would reduce the magnitude of anti-fetal immunity (Basu et al. 2008). As embryogenesis proceeds, autophagic activity becomes suppressed during the morula and blastocyst stages (Yue et al. 2003). The enhanced production of hsp70 during this time period suggests its possible involvement in decreasing the level of autophagy. The physiological significance of this possible interaction remains to be determined. It has been postulated that autophagy inhibition may reduce the rate of

lysosome-mediated degradation of fetal antigens and the subsequent expression of fetus-derived peptides on the surface of antigen-presenting cells (Kanninen et al. 2013a). This would reduce the extent of induction of maternal anti-fetal immunity. Regardless of the involved mechanism(s), it can readily be seen that exogenous or endogenous factors that alter hsp70 levels during early embryogenesis have the capacity to impinge on autophagy activity and may be detrimental to the early stages of embryo development.

The growth and differentiation of trophoblast cells and their migration into the maternal decidua to form the placenta require autophagy induction. In the first trimester, the blood supply to the placenta is low resulting in a limited availability of nutrients. Under these conditions, production of the enzyme 5′-adenosine monophosphate-activated protein kinase (AMPK) is induced. AMPK phosphorylates and inhibits activation of mTORC1, thereby inducing autophagy (Kim and Guan 2015). As gestation progresses, increased blood flow and oxygen availability results in elevated production of reactive oxygen species. This stimulates a further increase in autophagy to protect trophoblasts from apoptosis (Hung et al. 2013) and to maintain a sufficient supply of building blocks for protein, carbohydrate, lipid, and nucleic acid synthesis and placental development.

4 In Vitro Fertilization

For women undergoing in vitro fertilization, oocytes are fertilized and cultured in a dish for several days prior to the newly formed embryos being transferred to the uterus. These manipulations increase oxidative stress (Argarwal et al. 2006) as well as normal access to nutrients and cofactors. There is evidence that autophagy promotes in vitro embryo viability and function. Addition of the autophagy inducer, rapamycin, to the medium containing cultured bovine embryos was shown to improve in vitro blastocyst development (Song et al. 2012). In mouse embryos that were cultured in vitro for an extended time period, the induction of autophagy promoted survival (Lee et al. 2011). Interestingly, a recent study identified an association between the ability of sera from women undergoing an in vitro fertilization cycle to induce autophagy in peripheral blood mononuclear cells and the cycle outcome (Sisti et al. 2016a). A decreased ability to induce autophagy was associated with implantation failure following transfer of the embryos to the uterus, while a highly elevated autophagy level was associated with development of an extrauterine implantation (ectopic pregnancy). Thus, a proper level of autophagy induction by a factor(s) present in maternal serum promotes the survival and subsequent implantation of in vitro cultured embryos. Hsp70 is also induced under these conditions. In a mouse study, embryos cultured in vitro had higher levels of hsp70 than their in vivo counterparts (Tan et al. 2016). As mentioned above, heat shock protein expression was also shown to be required for the in vitro development of mouse embryos (Neuer et al. 1998). Further studies are needed to assess if maintaining a proper balance between autophagy and heat shock protein

production optimizes success in assisted reproduction. It would be interesting to determine whether addition of an autophagy inducer to in vitro cultured human embryos would improve reproductive outcome.

5 Pregnancy Complications

Preeclampsia is a common complication of pregnancy characterized by hypertension and proteinuria. Inadequate placentation results in development of excessive unopposed oxidative stress, release of antiangiogenic factors, and systemic inflammation, leading to placental and endothelial damage (Wu et al. 2015a, b). The influence of oxidative stress on the inhibition of mTORC1 and activation of autophagy in pregnant women has recently been reviewed (Ramos and Witkin 2016). Autophagy is increased above normal levels in the placenta in association with preeclampsia, perhaps to enhance the survival of trophoblast/ placental cells in this altered environment (Hung et al. 2013). The activation of autophagy by mTORC1 inhibition during gestation may also negatively influence fetal growth. Intrauterine growth restriction (IUGR), defined as a fetal weight below the 10th percentile for a given gestational age, is frequently associated with preeclampsia. In addition to being an inhibitor of autophagy, mTORC1 is also a central mediator in the transport of nutrients across the placenta (Jansson et al. 2012). Thus, inhibition of mTORC1, while perhaps being beneficial to trophoblast survival, may be detrimental to fetal development. As expected, hsp70 is also induced under these adverse conditions. Intracellular levels of hsp70 are higher in the placenta of women with preeclampsia than in normal controls (Park et al. 2014). The circulating hsp70 concentration is also elevated in women with preeclampsia as compared to controls (Molvarec et al. 2009). However, there is not necessarily a correlation between intracellular and extracellular hsp70 levels, and it is the intracellular hsp70 concentration that determines the extent of autophagic activity (unpublished observation). Therefore, the extent of autophagy inhibition by hsp70 in individual women with preeclampsia remains uncertain. In contrast to reports of increased placental autophagic activity in women with preeclampsia, sera from women with preeclampsia were shown to inhibit autophagy in peripheral blood mononuclear cells to a greater extent than did sera from normotensive pregnant women (Kanninen et al. 2014). IUGR is associated with some but not all cases of preeclampsia. It can be envisioned, therefore, that the greater the extent of hsp70 induction, the higher the level of inhibition of mTORC1 and the lower the level of both autophagy and mTORC1-mediated nutrient transport. Thus, variations in the extent of hsp70 induction in individual women may influence their severity of preeclampsia and its sequela.

Premature labor induction and delivery of a baby prior to term (<37 weeks gestation) is the major cause of neonatal morbidity and mortality. Inflammation due either to infection or to noninfectious (sterile) endogenous stimuli is a major cause of preterm birth (Romero et al. 2014). As would be expected, pregnancy-related

inflammation is accompanied by the increased production of hsp70 by fetal membranes (Menon et al. 2001) and elevated levels of hsp70 in the amniotic cavity (Chaiworapongsa et al. 2008), in the placental tissue (Chang et al. 2013), and in the maternal circulation (Fukushima et al. 2005; Chang et al. 2013). Hsp70-containing exosomes have also been identified in amniotic fluid of pregnant women (Asea et al. 2008), indicating that intercellular transport of hsp70 may occur during gestation. The increased induction of hsp70 in response to inflammation, as well as to other perturbations such as increased oxidative stress, would result in an inhibition of autophagy. In a mouse model, inhibition of autophagy was shown to result in induction of preterm birth, and this could be prevented by addition of rapamycin, an autophagy inducer (Hirota et al. 2011). In another mouse study, the decreased expression of autophagy-related genes in the uterus and placenta was associated with inflammation-mediated preterm labor (Agrawal et al. 2015). Levels of the autophagy inhibitor, mTORC1, were shown to be elevated in placentas from women who delivered preterm as compared to women with a term birth (Cha et al. 2013). Women positive for a genetic polymorphism (*ATG16L1*, rs2241880) that results in reduced autophagy activity have a more rapid progression from induced labor to delivery than do other women (Doulaveris et al. 2013). ATG16L1 is an inducer of Treg cells that suppress inflammation, and the polymorphic variant is associated with a hyper-pro-inflammatory immune response (Kabat et al. 2016; Chu et al. 2016). Thus, deficient Treg induction due to genetically related decreased autophagy activity can be seen to decrease immune tolerance and facilitate a more vigorous anti-fetal immune response that would increase the likelihood of premature parturition. Nuclear factor kappa B (NFκB), the transcription factor that activates genes coding for pro-inflammatory cytokines, is also an inhibitor of autophagy (Djavheri-Mergny et al. 2006). Hsp70 activation of NFκB (Asea et al. 2000) would thus serve to further potentiate autophagy inhibition. Another hsp70-autophagy interaction in parturition should also be noted. Autophagy promotes progesterone synthesis, an essential factor for maintaining uterine quiescence. The inhibition of autophagy coincides with induction of uterine contractions (Gawriluk and Rucker 2015). Hsp70 binds to the progesterone receptor interfering with progesterone binding and suppressing its activity (Bagchi et al. 1991). Thus, stress-induced hsp70 reverses uterine quiescence by inhibiting autophagy and by directly binding to the progesterone receptor. Interestingly, autophagy is increased (Sisti et al. 2016a, b) and hsp70 levels are decreased (Molvarec et al. 2007) in normal pregnancy as compared to the nonpregnant state. Autophagy facilitates cell survival during this period of rapid growth and differentiation as the fetus preferentially removes an increasing quantity of nutrients from the maternal circulation. Under these conditions, hsp70-induced inhibition of autophagy would limit the availability of nutrients and, therefore, jeopardize fetal growth.

We recently proposed a mechanism to explain how the inhibition of autophagy by hsp70 triggers parturition at term (Sisti et al. 2016a, b). The present paper expands on this model (Fig. 1). The extent of autophagy increases as gestation progresses to ensure that intracellular components no longer needed by the

differentiating maternal cells are removed and degraded to provide building blocks for new biosynthesis necessitated by the ever increasing removal of nutrients from the maternal to the fetal circulation. The intracellular accumulation of detrimental catabolic products such as reactive oxygen and nitrogen species would also decrease cell viability. Furthermore, the promotion of Treg induction by autophagy reduces inflammation to a level below which parturition would be triggered. Towards the end of the pregnancy, myometrial cells in the uterus become enlarged to such an extent (almost 100-fold) that both NFκB and hsp70, two inhibitors of autophagy, become activated. The decreased autophagy-related clearance of macromolecules as well as mitochondria and inflammasomes result in increased levels of reactive oxygen species and pro-inflammatory cytokines and limit the synthesis of new proteins that help to maintain myometrial cell quiescence. The loss of Treg induction further accentuates hyper-inflammation and a suppression of autophagy. Furthermore, as mentioned above, hsp70 interferes with progesterone activity by binding to the progesterone receptor. The net result is the initiation of myometrial contractions and the triggering of parturition.

6 Summary and Future Directions

The initiation of parturition by inhibition of autophagy strongly indicates that autophagy plays a central role in maintaining uterine quiescence. Conditions that are associated with preterm labor and delivery—infection, sterile inflammation, oxidative stress, physical and psychological trauma—all induce the production of hsp70. It is, therefore, reasonable to hypothesize that a trigger for premature parturition is the hsp70-mediated inhibition of autophagy. In parallel, the direct activation of mTORC1 by hsp70 results in an increased rate of transfer of nutrients to the fetus and, thereby, facilitates a more rapid development under circumstances that threaten continued gestation. The hsp70-mediated activities in pregnancy are summarized in Fig. 2. Further insight into this dichotomy—the positive and negative aspects of hsp70 induction in pregnancy—has the potential to lead to development of novel protocols to reduce the likelihood of hsp70-mediated premature delivery as well as to maximize fetal nutrition under conditions where the length of gestation is being threatened. Potential avenues for future study include an analysis of the influence of exogenous autophagy inducers on preterm parturition and neonatal well-being, identification of compounds that increase tolerance to infectious agents or other inducers of the stress response, and detection of selective inducers of mTORC1-mediated placental transport of nutrients that do not inhibit autophagy.

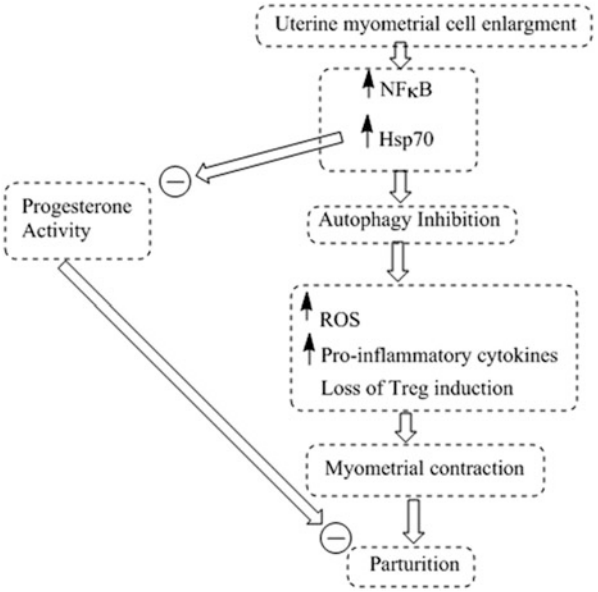

Fig. 1 Involvement of hsp70 and autophagy in the initiation of parturition. At term the uterine myometrial cells become greatly enlarged and high levels of hsp70 as well as NFκB are induced. Both hsp70 and NFκB inhibit autophagy leading to increased intracellular accumulation of reactive oxygen species (ROS), enhanced pro-inflammatory cytokine production and a loss of Treg induction. These factors lead to a decrease in factors that promote uterine quiescence and, as a result, myometrial contractions are induced. Concomitantly, hsp70 inhibits progesterone binding to its receptor further stimulating myometrial contractions. The net result is the initiation of parturition

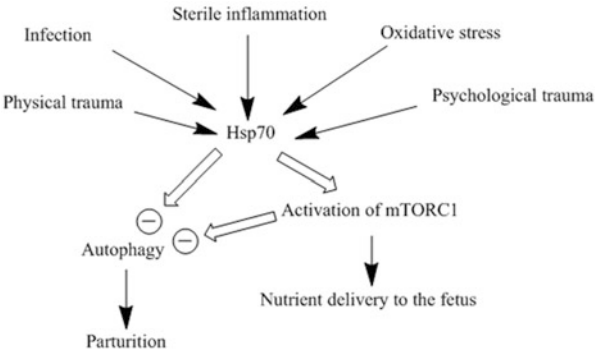

Fig. 2 Involvement of hsp70 in pregnancy. Any one of a multitude of stresses leads to the induction of hsp70. High levels of hsp70 inhibit autophagy and thereby promote parturition either prematurely or at term. Concomitantly, elevated hsp70 promotes the activation of mTORC1, the major inhibitor of autophagy, but also an essential factor for the transport of nutrients from the maternal to the fetal side of the placenta

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Roles of Grp78 in Female Mammalian Reproduction

Cheng Zhang

Abstract The glucose-regulated protein (GRP78) also referred to as immunoglobulin heavy chain binding protein (Bip) is one of the best characterized endoplasmic reticulum (ER) chaperone proteins, which belongs to the heat-shock protein (HSP) family. GRP78 as a central regulator of ER stress (ERS) plays many important roles in cell survival and apoptosis through controlling the activation of transmembrane ERS sensors: PKR-like ER-associated kinase (PERK), inositol requiring kinase 1 (IRE1), and activating transcription factor 6 (ATF6). Many studies have reported that GRP78 is involved in the physiological and pathological process in female reproduction, including follicular development, corpus luteum (CL), oviduct, uterus, embryo, preimplantation development, implantation/decidualization, and the placenta. The present review summarizes the biological or pathological roles and signaling mechanisms of GRP78 during the reproductive processes. Further study on the functions and mechanisms of GRP78 may provide new insight into mammalian reproduction, which not only enhance the understanding of the physiological roles but also support therapy target against infertility.

Abbreviations

ATF6	Activating transcription factor 6
Bip	Immunoglobulin heavy chain binding protein
CHOP	C/EBP-homologous protein/DNA damage-inducible transcript 3
CL	Corpus luteum
eIF2α	Eukaryotic translation initiation factor 2α
ER	Endoplasmic reticulum
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
GRP78	Glucose-regulated protein
HSP	heat-shock protein

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IRE1	inositol requiring kinase 1
JNK	c-Jun N-terminal kinase
LH	Luteinizing hormone
PERK	PKR-like ER-associated kinase
UPR	Unfolded protein response

1 Introduction

Endoplasmic reticulum (ER) is an important cytoplasmic compartment, which synthesizes membrane proteins and lipids, and maintains Ca^{2+} homeostasis in eukaryotic cells. The physiological functions of ER are essential for the survival of eukaryotic cells (Luo et al. 2006). Once the ER homeostasis is disturbed under stress conditions, the unfolded proteins are accumulated and the unfolded protein response (UPR) is induced. Subsequently, three ER-resident transmembrane proteins: PERK (PKR-like ER-associated kinase), IRE1 (inositol requiring kinase 1), and ATF6 (activating transcription factor 6) are activated to act as transducers of ER stress signaling, which in turn relieve ER stress (ERS) and promote cell survival. During the UPR, a cascade of processes is induced to maintain the ER homeostasis. Firstly, the newly synthesized proteins are reduced by transient arrest of protein translation; secondly, the folding capacity is increased via upregulating ER gene expression; thirdly, the misfolded and unfolded proteins are degraded to reduce the burden of the ER. However, the cellular apoptotic pathways are induced when the ER stress is overloaded. The activation of CHOP (C/EBP-homologous protein), JNK (c-Jun N-terminal kinase), and caspase-dependent pathway are also involved in the apoptosis process (Lee 2005; Quinones et al. 2008).

The glucose-regulated protein (GRP78) is one of the best characterized ER proteins. GRP78 is a 78,000 Da protein, which was originally named in 1977 since the protein level in chick embryo fibroblasts (CEF) was increased after the rapid depletion of glucose in the medium (Shiu et al. 1977). GRP78 is also commonly regarded as the immunoglobulin heavy chain binding protein (BiP); the latter was first detected to non-covalently bind to the immunoglobulin heavy (IgH) chains of Pre-B cells (Haas and Wabl 1983). GRP78 is a member of the HSP70 family of proteins and known as heat shock 70 kDa protein 5 (HSPA5) since it shares partial amino acid sequence identity with HSP70, the 70-kDa heat shock protein. GRP78 consists of three domains: a 44 kDa ATPase domain in the N-terminal domain, a 20 kDa polypeptide-binding domain in the C-terminal, and a 10-kDa domain of unknown function in C-terminal tail (Chevalier et al. 2000).

It is well known that GRP78 is localized in ER lumen. In addition, many studies have showed that GRP78 is also presented on the cell surface where it acts as receptor-like function and regulates cellular proliferation and survival (Gonzalez-Gronow et al. 2009; Sato et al. 2010; Ni et al. 2011; Gray and Vale 2012). In non-stressed cells, GRP78 is mainly located in the perinuclear ER associated with its C-terminal retention signal. GRP78 plays important roles in many cellular

processes as an ER molecular chaperone including translocating newly synthesized polypeptides across the ER membrane, guiding the newly synthesized protein folding and assembly, and targeting misfolded proteins for degradation in ER. Meanwhile, GRP78 maintains intracellular Ca^{2+} homeostasis by preventing the depletion of ER intracisternal Ca^{2+}. GRP78 also regulates the process of Ca^{2+} efflux from ER to mitochondria, which affects bioenergetics and sustains cellular survival (Lee 2001; Hendershot 2004). Additionally, GRP78 also binds to PERK, IRE1, and ATF6 in the ER membrane as a complex to maintain the sensor molecules in an inactive form. However, stress, like changed environmental and physiological conditions, as well as pharmacological reagents affecting protein folding, glycosylation, or the ER calcium pool, may induce UPR in the ER. GRP78 is initiated and involved in the signaling cascade that results in the UPR, which leads to dissociation of GRP78 from the above three complex. And then, PERK, IRE1, and ATF6 are activated and act as transducers of ER stress signaling. Activated PERK, IRE1, and ATF6 positively regulate ER chaperone proteins, folding enzymes, and protein degradation molecules via joint or independent ways. ERS-UPR cascades either prevent the accumulation of unfolded proteins or increase their subsequent folding. Meanwhile, the cascades also promote the degradation of excessive misfolded proteins (Fig. 1) (Yung et al. 2008; Burton et al. 2009; Wang et al. 2012; Yang et al. 2015a).

Since PERK has a kinase domain, activated PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α). The latter transiently suppresses the

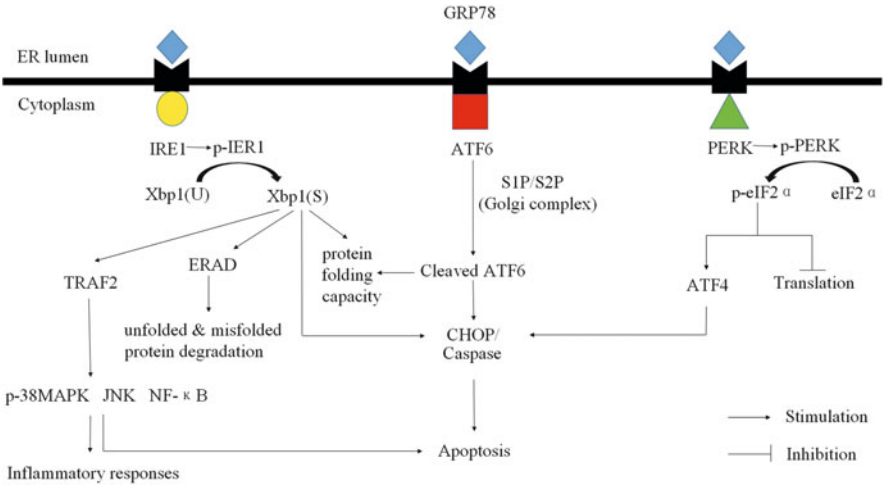

Fig. 1 Schematic diagram showing the signaling of GRP78 in ER stress (ERS) and unfolded protein response (UPR). In non-stressed cells, GRP78 interacts with PERK, IRE1, and ATF6 in the ER membrane as a complex to maintain the sensor molecules in an inactive form. Upon ERS, all three sensors are released from GRP78 and triggering the UPR which aims to restore homeostasis. Once the original stress is not resolved, the pro-apoptotic phase is initiated involving a subsequent cross talk between the ER and mitochondria. GRP78 regulates the balance between cell survival and apoptosis in ER-stressed conditions through the above interactions

translation process of most de novo proteins and reduces the inflow of nascent proteins into the ER lumen (Harding et al. 2000). However, the translation of transcriptional factor ATF4 is activated via internal ribosomal entry site. And then CHOP, a downstream target of ATF4, is involved in the translation and apoptosis (Marciniak et al. 2004; Li et al. 2008).

IRE1's endonuclease activity is released after its activation, which generates spliced mRNA of the XBP1 (X-box binding protein). The latter activates transcription of genes, which regulate the degradation of unfolded and misfolded proteins through ER-associated degradation (ERAD), as well as ER biogenesis (Kaufman 1999, 2002; Gonzalez-Gronow et al. 2009). IRE1 can also induce proinflammatory response by MAPK (mitogen-activated protein kinase), JNK (c-Jun N-terminal kinase), and NF-kB (Nuclear factor kB) via binding its kinase domain (Burton et al. 2009; Fu et al. 2015). In addition, JNK as the downstream target of IRE1 is also involved in regulating cell death (Urano et al. 2000; Lei and Davis 2003; Li et al. 2008).

Activated ATF6 translocates from the ER to the Golgi complex and then is cleaved by S1P (site 1 protease) and S2P (site 2 protease). The other active transcriptional factor, p50ATF6 fragment, is generated. The p50ATF6 enters the nucleus and increases the expression of UPR target genes, including Grp78 by ERSE (ER stress response element) in the promoter region of the GRP78 gene (Kaufman 1999, 2002; Ye et al. 2000; Shen et al. 2002; Hong et al. 2004; Lee 2005).

Taken together, two phases may be executive during UPR process (Fig. 1) (Lee 2005; Burton et al. 2009; Feng et al. 2014; Fu et al. 2015; Yang et al. 2015a). First phase is that GRP78 acts as a pro-survival factor. These processes are important to increase the protein folding capacity and attenuate the biosynthetic burden of ER during ER stress. GRP78 plays important roles in these processes, especially in the cascade of IRE1 and ATF6 for preventing ER stress-induced apoptosis (Yoshida et al. 1998; Gonzalez-Gronow et al. 2009). If the original stress is not resolved, the pro-apoptotic phase is initiated involving a subsequent cross talk between the ER and mitochondria (Rutkowski and Kaufman 2004; Szegezdi et al. 2009). Therefore, GRP78 is an important modulator of ER stress transducers.

GRP78 is not only localized in the lumen of the ER but also in the surface of specific cell types. GRP78 plays an important role in regulating the UPR as an ER molecular chaperone. Many studies have reported that GRP78 is involved in the physiological and pathological process in female reproduction, including follicular development, corpus luteum (CL), oviduct, uterus, embryo, preimplantation development, implantation/decidualization, and the placenta. The present review focuses on the expression and function of GRP78 in female mammalian reproduction.

2 Expression and Effect of Grp78 in Female Mammalian Reproduction

2.1 The Roles of Grp78 in Ovary

Mammalian ovarian follicular development is a complicated and delicate process, which is tightly regulated by endocrine, autocrine, and paracrine factors. Ninety nine percent of follicles eventually undergo atresia, a process involving granulosa cell apoptosis. The survival of granulosa cells in growing follicles is due to the balance between survival factors and cell death inducers (Zhang et al. 2011, 2013; Guan et al. 2015). GnRH is the main regulator of the hypothalamic–pituitary–gonadal axis, which stimulates the pituitary gonadotroph to synthesize and secrete Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) (Zhang et al. 2012a). FSH is a dominant factor that promotes follicular development during the pre-ovulatory stage by the cross talk among reproductive hormones, like estradiol, progesterone, testosterone, gonadotrophin releasing hormone (GnRH), and so on. Along with follicle growth, FSH induces LHR expression in the granulosa cells of pre-ovulatory follicles. The ovulation is trigged by the LH surge, and the process of ovulation has been considered an inflammation-like phenomenon (Espey and Richards 2002; Richards et al. 2002; Iwamune et al. 2014). Once the oocyte is released from mature follicle, the leftover cells, especially granulosa cells, form the corpus luteum which synthesizes and secretes progesterone for preparing and to sustain pregnancy. The hypothalamic–pituitary–gonadal axis plays critical role in the whole process of follicular development.

In previous reports, the GRP78 expression is detected in the ovary of different species, including rat (Kogure et al. 2013), mouse (Yang et al. 2013), goat (Lin et al. 2012b), and cow (Cree et al. 2015). GRP78 is mainly expressed in granulosa cells in healthy follicles (Lin et al. 2012b; Kogure et al. 2013; Yang et al. 2013). Meanwhile, the mRNA level in granulosa cells is also detected with lower level pattern in healthy follicles. Equine chorionic gonadotropin (eCG) which including FSH and LH fails to significantly change GRP78 expression although it mainly exerts FSH function. However, human chorionic gonadotropin (hCG), which mainly exerts LH function, significantly upregulates GRP78 mRNA content at 6 h after ovulatory dose of hCG injection in rat ovary. The upregulation reaches to peak at 12 h after treatment and then recover to the normal level during the transformation of the ovulated follicles into the corpus luteum 48 h after hCG injection. The increased mRNA content of GRP78 may account for the transient peak of GRP78 protein level at 18 h after hCG injection (Kogure et al. 2013).

It is very interesting that expression pattern of LHR mRNA is opposite to the pattern of GRP78. LHR mRNA level is the lowest at 12 h after hCG treatment and gradually increased from 24 h after hCG injection. In addition, GRP78 protein expression preceded the cell surface LHR binding sites after the downregulation by

rGRP78 siRNA. Moreover, GRP78 increases LHR expression both inside the cell and at the cell surface in HEK-293 cells (Kogure et al. 2013). It is possible that the transient increase of GRP78 by hCG induces LHR expression in granulosa cells in the early stage of luteinization through enhancing the promoter activity of GRP78. The increased LHR expression is very important to the formation of corpus luteum (CL). However, why and how does hCG regulate GRP78 expression in granulosa cells? The possible mechanism is that induced miR-376a by hCG impacts GRP78 expression since miR-376a binds to the 3′-end of GRP78 mRNA from 2439–2459 bp in the 3′-UTR. Due to 44 miRNAs that can bind to the 3′-UTR of GRP78 mRNA by the MicroCosm analysis of miRNA targets, a network among multiple miRNAs is possible to be involved in the regulation of GRP78 mRNA (Iwamune et al. 2014).

In atresic follicles of goat, the expression GRP78 is increased with the condition of atresia. Meanwhile, the GRP78 expression is also along with the elevated DDIT3 (DNA damage-inducible transcript 3, which is also called CHOP), ATF4, and ATF6 expression in the granulosa cells from atresic follicles (Lin et al. 2012b). It is surprising that there is no positive staining for GRP78 in the apoptotic granulosa cells from mature mouse (Yang et al. 2013). In the physiological condition, the expression pattern of GRP78 may depend on the species. However, once the cultured system of granulosa cell in vitro is changed by serum withdrawal or pharmacological agents like tunicamycin (Tm), a specific inhibitor of N-glycosylation that blocks asparagines (N)-linked glycosylation by forming N-glycosidic protein–carbohydrate linkages, and thapsigargin (Tg), which blocks ER uptake of calcium by inhibiting the sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA) (Mahoney and Duksin 1979; Zinszner et al. 1998), the percentage of apoptotic cells is increased. Meanwhile, GRP78 expression is dramatically increased along with CHOP, ATF4 and ATF6, and caspase-12 expression. In these apoptotic cases, the upregulation of GRP78 fails to prevent granulosa cells from apoptosis by the initiation of UPR signal transduction pathways. In contrast, apoptotic cells are dramatically increased. The possible explanation is the ERS by serum withdrawal or pharmacological agent is persistent or excessive and ER homeostasis cannot be reestablished. The apoptotic response may be initiated by transcriptional induction of CHOP/ATF4, the ER-associated caspase-12 pathway, and/or the activation of the JNK-dependent pathway (Kim et al. 2006; Rasheva and Domingos 2009; Shore et al. 2011; Lin et al. 2012b).

In addition, the excessive intracellular lipid content in the cumulus–oocyte complexes (COCs) is associated with cumulus cell apoptosis, which may be induced by the lipotoxicity. It is well known that high levels of free fatty acids and lipid peroxides change the structure and function of ER (Borradaile et al. 2006; Diakogiannaki et al. 2008), which lead to accumulation of unfolded proteins and calcium release. ERS-mediated cumulus cell apoptosis is supported by the results that the expressions of GRP78 and ATF4 in COCs from high-fat diet-fed mouse are remarkably upregulated (Wu et al. 2010). Moreover, the culture system for COCs

in vitro with lipid-rich follicular fluid impairs oocyte maturation, which is also related with ERS including GRP78 (Yang et al. 2012). The administration of FSH increases GRP78 protein expression in oocytes in old cow, which indicates ERS possibly impair oocyte quality following stimulation. It is a possible explanation for the lowering oocyte quality with advancing maternal age, especially under the hormone stimulation in the clinic (Cree et al. 2015).

The CL is a transient, rhythmic endocrine organ that develops after ovulation from the ruptured follicle during the luteal phase. The granulosa cells turn into luteal cells by proliferation and differentiation. High level of progesterone (P_4) is secreted by the CL and blocks gonadotropin secretion and restricts follicular development. Progesterone is also associated with the regulation of the CL life span, which is very important to establish and maintain the pregnancy in mammals (Choi et al. 2011; Yang et al. 2015a). However, the CL regresses and a new ovulation cycle restarts if pregnancy does not occur (Jiemtaweeboon et al. 2011). Many studies have reported that cellular apoptosis in the CL is detected during spontaneous and induced regression in many species, including rats (Bowen et al. 1996; Gaytan et al. 2000; Telleria et al. 2001), cow (Juengel et al. 1993; Rueda et al. 1997), sheep (Rueda et al. 1995), and humans (Shikone et al. 1996). Previous studies have suggested that both death-receptor- and mitochondria-mediated apoptotic systems are active in the CL regression (Quirk et al. 2000; Carambula et al. 2003; Dauffenbach et al. 2003). In addition, autophagy has also been identified in CL regression (Choi et al. 2011). Although apoptosis is involved the apoptotic progress of CL (Stocco et al. 2007), the precise mechanism of regulating the regression of the CL is not fully understood.

Recently, several reports have shown that ERS play important roles in the formation, maintenance, and regression of CL (Lai et al. 2007; Park et al. 2013b). It has been reported that UPR signaling is involved in the maturation of secretory cells including antibody-producing plasma cells, osteoblasts that secrete collagen, and insulin-secreting pancreatic β-cells (Wu and Kaufman 2006). In addition, UPR plays an important role in steroidogenic enzyme expression by modulating the ATF6 pathway in hCG-stimulated Leydig cells (Park et al. 2013b). Moreover, the CL needs steroidogenic enzymes to produce progesterone. It is possible that UPR, especially ATF6 pathway, may also play an significant role in regulating steroidogenic enzyme expression during the estrous cycle (Park et al. 2013a, 2014).

In cow, increased Grp78 expression as a major UPR regulator is detected during the early stage and mid-stage of the CL. Then the rapidly decreasing Grp78 expression occurs during the regression stage, which is accompanied by increased caspase 3 cleavage. These results indicate that GRP78 is very important to the CL life span in the estrous cycle in bovines. Furthermore, three signaling pathways eIF2α/ATF4/GADD34 (growth arrest and DNA-damage-inducible gene34), p90ATF6α (90-kDa ATF6α)/p50ATF6α (90-kDaATF6α), and IRElα/XBP1 are found to be involved in the luteal phase progression during the estrous cycle (Park et al. 2013a). These results indicated that Grp78/Bip, ATF6, and XBP1 act

as ER chaperones to form and maintain the CL and block caspase activation during the early and mid-stages of the CL. During the CL regression stage, prolonged ER stress leads to CHOP expression and JNK phosphorylation by the activation of the PERK/eIF2a/AFT4 and IRE1 pathways via ER stress-mediated pro-apoptotic signaling, respectively (Lai et al. 2007). In turn, pro-apoptotic caspase 3 is cleaved and activated, thereby leading to apoptosis (Jing et al. 2012). Similar results are also reported in mouse (Park et al. 2014). Hyo-Jin Park et al. found that UPR signaling pathways activated in response to ER stress may play important roles in the regulation of the CL function during the CL life span. Yang's results further confirm that ERS-mediated apoptosis regulates the regression of rat CL through the CHOP pathway and caspase-3/12 (Yang et al. 2015b). In addition, PGF2-α-induced CL apoptosis is accompanied by increased GRP78 expression by PGF2α. However, tauroursodeoxycholic acid (TUDCA), an endogenous hydrophilic bile acid, acts as a chemical chaperone and attenuates ERS, decreases GRP78 expression, and inhibits CL apoptosis (Yang et al. 2015b).

Taken together, GRP78 is mainly expressed in granulosa cells with low-level staining in healthy follicles, which is regulated by hCG. However, the expression patterns of GRP78 in atresic follicle are different in different species in physiological conditions. UPR signaling pathway is involved in the apoptotic process of granulosa cells. Meanwhile, GRP78 is also involved in oocyte maturation although the mechanism is not clear. Moreover, GRP78 is highly expressed in early and mid stage of the CL and then decreased GRP78 expression is accompanied with the CL regression. ERS play an important role in regulating CL formation, regression, and function through the three UPR signaling pathways. Grp78 may act as an ER chaperone and an inhibitor of caspase activation during follicular development, ovulation, and CL formation and maintenance during the estrous cycle. Once ER stress is excessive or persistent, ER-stress-mediated apoptotic factors are activated and the UPR signaling pathway is initiated and promotes cell apoptosis. However, the specific and delicate mechanism still remains unknown in the follicle and CL development. Further study will be needed to investigate the cross talk and network that exists among death-receptor-, mitochondria-, ERS-mediated apoptotic signaling, and autophagic cell death system during follicular development and CL life span.

2.2 Bioregulatory Role of Grp78 in the Oviduct

The mammalian oviduct is a dynamic organ, which plays a critical role in the reproduction events leading to the establishment of pregnancy by providing a stable and optimal microenvironment. These events include final maturation and transport of the female and male gametes, fertilization, initial stages of embryonic development, and transport of the embryo to the uterus (Peng et al. 2015). The physiological

interaction and cross talk between gametes, embryo, and oviductal epithelia affect the above events in an intimate and specific way. The presence of gametes in the oviduct induces modification of oviductal gene transcription (Fazeli et al. 2004; Georgiou et al. 2005). Since the transportation of sperm to the oviduct may occur several hours before ovulation, this tube-shaped organ plays an essential role in accomplishing the fertilization process. Once the oviductal epithelia attach to the sperm, the viability, motility, and capacitation are dramatically regulated (Sostaric et al. 2006). Meanwhile, the amounts of live and competent sperms are released to the oocyte for fertilization whenever the ovulation occurs (Hunter and Rodriguez-Martinez 2004).

Moreover, the oviduct epithelium is also essential for the oocyte cytoplasmic maturation, subsequent blastocyst cell proliferation (Kidson et al. 2003) and embryo development (Hill 2001). A number of plasma membrane molecules in oviductal epithelia have been detected, which are important to understand the function of this tube during the reproductive process (Sostaric et al. 2006). Meanwhile, it is necessary to know how the proteins respond to a variety of intracellular and extracellular signals within the oviduct. It has been reported that oviduct epithelial cells (OEC) interacts with and attaches to mammalian sperm during sperm transit along the female reproductive tract (Ellington et al. 1993, 1999a, b; Gualtieri et al. 2005; Lachance et al. 2007). Moreover, only higher-quality spermatozoa are selected to bind OEC (Ellington et al. 1999a). Cocultured with OEC, the sperm viability, motility, and acrosomal integrity are all improved in bovine (Boilard et al. 2004), which indicate that either factors from OEC trigger the intracellular events on spermatozoa or factors strongly attached to sperm cells act in positive effects.

Further investigation finds that GRP78 is one of the factors expressed in the bovine oviduct epithelial cell surface, which positively affects capacitation of sperm by binding to the spermatozoa. Although GRP78 fails to directly affect sperm viability, motility, or acrosomal integrity in human oviduct, expressed GRP78 by OEC binds to human spermatozoa and modulates sperm capacitation via phosphorylating protein tyrosine and intracellular calcium levels during sperm passage through the female reproductive tract and/or during its contact with oviduct epithelial cells (Lachance et al. 2007). Moreover, Grp78 binds to the sperm acrosomal cap and prevents the number of spermatozoa bound to the zona pellucida (ZP) in a calcium-dependent manner. It is interesting that soluble Grp78 is also detected in oviductal fluids in the periovulatory period (Boilard et al. 2004). The possible reason for this is that the protein contains a signal peptide and might be expressed extracellularly with one putative transmembrane domain according to the amino acid sequence of human GRP78 (Boilard et al. 2004). The soluble Grp78 in this particular phase may facilitate spermatozoa's acquisition of the ability to interact with and fertilize the oocyte (Marin-Briggiler et al. 2010).

During the estrous cycle, the mammalian oviduct undergoes significant endocrine-induced changes in morphology, biochemistry, and physiology, which

establish an essential microenvironment within the oviduct for interaction with gametes and embryos (Peng et al. 2015). Whether the expression of GRP78 is also different during the whole phase of estrous cycle is not very clear in different species. Recently, Lin's research finds that GRP78 is mainly expressed in epithelial cells, especially in the cilia of ciliated cells, which is consistent with the previous report in human (Lachance et al. 2007; Lin et al. 2012a). However, the expression pattern is diverse in different areas of oviduct. GRP78 is highly expressed in the epithelial cells of isthmus compared with those of infundibulum and ampulla. Furthermore, the expression and distribution in the oviducts varied with the phase of the murine estrous cycle. Highly positive staining for GRP78 is observed during the estrus phase compared with proestrus, metestrus, and diestrus (Lin et al. 2012a). The protein expression of GRP78 may account for the transcriptional regulation since mRNA levels of GRP78 is also highest at estrus (Lin et al. 2012a). The estrus is a particular phase of reproductive cycle since gametes are transferred to the female reproductive tract at this phase of the cycle, and fertilization occurs under normal conditions. The elevated GRP78 levels in the oviducts may play many important roles, such as gamete transport, sperm capacitation, and early embryogenesis. In addition, no positive staining for GRP78 is detected in mouse stromal cells although GRP78 is expressed by oviduct stromal cells in human (Lachance et al. 2007; Lin et al. 2012a).

In summary, GRP78 is mainly expressed in oviduct epithelial cells in different species. The expression pattern is varied with the reproductive cycle. In addition, the soluble GRP78 is present in human oviductal fluids in the periovulatory period. GRP78 acts as a factor secreted from oviduct epithelial cells, which positively affects capacitation of sperm by binding to the spermatozoa. The sperm binding to oviduct epithelium is considered to be a key step in generating a highly fertile capacitated sperm population primed for fertilization in many species. Future studies should be directed toward understanding the potential function and delicate mechanism of GRP78 in the oviduct epithelium cell in different stages of the cycle. This knowledge would help us to further understand the critical events taking place in oviduct between gametes/embryo and reproductive tract, which are important to the conception and establishment of pregnancy.

2.3 Effect of Grp78 in the Uterus

The uterus is a major female hormone-responsive reproductive sex organ of most mammals, which includes three layers: endometrium, myometrium, and peritoneum from innermost to outermost. The endometrium is a steroid-sensitive and dynamic tissue that undergoes cyclic regeneration, including tissue differentiation, proliferation, apoptosis, and angiogenesis under the influence of estrogen and progesterone, as well as numerous local paracrine and autocrine factors, during

each menstrual cycle (humans) or estrous cycle (most other mammals) (Kayisli et al. 2002). Eventually, the endometrial secretory lining provides a hospitable environment for blastocyst implantation. There are many confirmations that heat shock proteins (HSP) are detected and involved in the regulation of steroid-responsive endometrium (Tabibzadeh et al. 1996; Tabibzadeh and Broome 1999; Reese et al. 2001).

The presence of GRP78 in uterus has been previously reported in different species (Simmons and Kennedy 2000; Lachance et al. 2007; Guzel et al. 2011; Lin et al. 2012a, 2014). In human, GRP78 is mainly expressed in the endometrial glandular and stromal cells (ESC). The results of immunolocalization find that GRP78 is dominantly expressed in mice endometrial glandular epithelial cells, which is consistent with the results in human and rat (Simmons and Kennedy 2000; Lachance et al. 2007). However, no positive staining of GRP78 is detected in the myometrium and the ESC at different phases of estrous cycle in mice (Lin et al. 2012a).

The studies show that the GRP78 expression is cycle-dependent. In human, GRP78 immunoreactivity in endometrial glandular cells in the early proliferative (Days 1–5 of menstrual cycle), early secretory (Days 15–18 of menstrual cycle), and late secretory phases (Days 24–28 of menstrual cycle) is significantly higher than in other phases. In ESC, GRP78 expression is significantly higher in the early proliferative phase and the late secretory phase compared to all other cycle phase (Lachance et al. 2007; Guzel et al. 2011). Moreover, GRP78 expression and distribution in the mice uterus also vary with the phase of estrous cycle. The mRNA level of GRP78 is highest at estrus than other phases, which is consistent with GRP78 protein expression pattern.

The menstrual or estrous cycle-dependent changes in the amounts of GRP78 suggest the regulations are associated with alterations in steroid hormones. One possible reason is that estrogen-induced GRP78 expression in the mouse uterus is an ER-independent manner as a phase I response (Ray et al. 2006). Moreover, estradiol (E2) significantly decreases tunicamycin-induced GRP78 expression in Ishikawa cells (a well-differentiated endometrial adenocarcinoma cell line) in vitro (Guzel et al. 2011). Additionally, the expression of HSP70 has been shown to be correlated with sex steroid receptors, and amounts of receptors are known to exhibit menstrual cycle-dependent changes in female endometrium (Koshiyama et al. 1995; Tabibzadeh et al. 1996). In addition, it is well known that the synthesis and secretion of steroid hormones are regulated by FSH and LH during the cycle. It has been reported that GRP78 regulates FSHR and LHR expression since gonadotropin receptors can be associated with chaperone proteins in the ER (Mizrachi and Segaloff 2004). The cross talks among reproductive hormones and GRP78 are possibly involved in the functional and structural cycle-dependent changes in the uterus.

Several lines of evidence indicate that GRP78 plays an important role in the implantation of embryo. It is well known that the implantation is the first step to

establish an intimate connection between the uterus and developing conceptus. Only a receptive uterus can accept the implantation of mammalian embryo and the receptive uterus only lasts a short period in pregnancy. The implantation is initiated by the embryo trophectoderm to attach to the uterine epithelium and then invade the endometrium (Kimber and Spanswick 2000; Dey et al. 2004; Hayashi et al. 2009). Uterine receptivity is closely related to optimal sensitization for the decidualization (De Feo 1963). E_2 and P_4 are critical hormones for the implantation process (Hayashi et al. 2009). Exposure to E_2 or P_4 can induce a prereceptive "neutral" state or refractoriness state, respectively. Both states become hostile to blastocysts (Psychoyos 1986). Herein, a time-dependent progesterone-estrogen sequence is very important to induce an endometrial receptive/sensitized state, which is a transient event window between the neutral and refractory states (Simmons and Kennedy 2000). Uterine receptivity/sensitization is induced by the combination effect of P_4 priming followed by exposure to a specific concentration of E_2 (Psychoyos 1973). Intermediate E_2 increased endometrial GRP78 mRNA and protein levels on day 5 of pseudopregnancy in ovariectomized animals in vivo (Simmons and Kennedy 2000; Lin et al. 2014). The increased GRP78 within the endometrium during the uterine sensitization indicates that hormones can regulate its expression. The expression of GRP78 is significantly increased in the luminal and glandular epithelium on day 5 of early pregnancy and gradually increased with the process of decidualization (Lin et al. 2014). Moreover, on day 5 of embryo implantation in rat, the uterus has a "receptive endometrium," and GRP78 mRNA content in endometrium is dramatically increased during the stage of decidualization. Meanwhile, the expression of mRNA is mirrored by the protein changes (Simmons and Kennedy 2000). Thus, GRP78 might play a role in preparing a receptive endometrium.

Further study shows that the GRP78 protein is weakly expressed during the early pregnancy, and the signal is gradually vanished in the uterus of pseudopregnancy compared with the normal pregnancy (Lin et al. 2014). Moreover, GRP78 expression depends on the presence of active embryo implantation since GRP78 protein content in implantation sites is higher than that at other sites without embryos (Lin et al. 2014). These results indicate that GRP78 expression at implantation sites might be induced by active blastocysts or maternal hormones. It has been reported that the rise in protein within the endometrium occurs on day 3 of pregnancy, followed by a decrease on day 4 and a rise again on day 5 after which it remains high in implantation sites (Reid and Heald 1970, 1971). In several cell types, the secretory apparatus size is increased and accompanied by increased secretory workload, which creates the need for more resident ER proteins (Wiest et al. 1990). The increased GRP78 during the embryo implantation is possibly necessary for the protein synthesis by assisting in the folding and processing of proteins in the ER lumen.

It is well known that GRP78 is normally induced under conditions of stress, such as glucose deprivation, Ca^{2+} depletion, and oxygen deprivation, which inhibit

protein folding and maturation within the ER. During the process of blastocyst formation and implantation, intrauterine oxygen tension in the hamster decreases under estrogenic stimulation (Fischer and Bavister 1993). The mild oxidative stress (OS) can induce GRP 78 expression in the normal human decidual cells (Gao et al. 2012). Additionally, the homeostasis of calcium (Ca^{2+}) in the uterus is critically important for embryo implantation, and Ca^{2+} transport genes are abundantly expressed in reproductive tissues in a distinct manner. ERS is triggered by a disturbance of Ca^{2+} homeostasis, which plays an important role in the maintenance of Ca^{2+} homeostasis followed the UPR (Park et al. 2010; Dai et al. 2012). The successful blastocyst implantation is related to the upregulation of the calcium-binding protein S100P (Tong et al. 2010; Zhang et al. 2012b) and S100A11 (Liu et al. 2012) in human endometrium. These results suggest that increased Grp78 in the pregnant uterus plays important roles in embryo implantation through the UPR and ERS.

In contrast, the investigation in several cell lines show that expression of GRPs as ER chaperones is dependent upon and regulated by a mitogenic pathway by some growth factors, which is distinct from the stress-inducible UPR cascade (Brewer et al. 1997). In rat endometrium, GRP94 is not induced concurrently with GRP78 during uterine sensitization as a chaperone coregulated with GRP78 in a general response to cellular stresses (Little et al. 1994). This partially suggests that the attainment of a receptive state is mediated not only by activated cellular stress pathway but also via a novel, uncharacterized pathway.

In summary, GRP78 is dominantly expressed in endometrial glandular epithelial cells in different species and the expression pattern is cycle-dependent. The expression of GRP78 is highest at estrus than other phases. However, the expression of GRP78 in the endometrial stromal cells depends on the species. The cross talks among reproductive hormones and GRP78 are possibly involved in the functional and structural cycle-dependent changes in uterus. The increased GRP78 is necessary to the uterine receptivity/sensitization and decidualization, which are important to the embryo implantation. The excessive ERS could influence proper function of UPR and lead to cell damage as well as inhibition of cell growth and activation of apoptosis. Eventually, implantation failure and early pregnancy loss are induced. Further studies are needed to investigate the delicate mechanisms involved in the induction of GRP78 in uterus during the embryo implantation.

2.4 Involvement of GRP78 in Embryonic Development

Recent studies show that UPR plays many important roles in the development of embryo at all stages. The major components of the ERS-UPR cascade are present at all stages of preimplantation development. GRP78 is minimally detected in zygotes and weakly detectable from the two-cell stage to the morula stage, and its abundant

expression is found at the blastocyst stage in the developing mouse embryo (Kim et al. 1990). Furthermore, the major UPR constituents including GRP78 are present at all stages of mouse preimplantation development, and activated XBP1 arm of the ERS is involved in the developmental period (Abraham et al. 2012). It is not surprising that Grp78 is presented throughout preimplantation development and constitutively expressed in most tissues. ERS-induced GRP78 represents a major prosurvival arm of the unfolded protein response (UPR) during the stage of preimplantation development. Previous reports have shown that GRP78 has an essential physiological effect for embryonic cell proliferation and protects the inner cell mass (ICM) from apoptosis during early stage of developing mouse embryos (Kim et al. 1990).

Based on knockout mouse models, the cell replication of ICM is substantially reduced in GRP78$^{-/-}$ (homozygous knockout) embryos. Moreover, the homozygous Grp78 null embryos fail to survive further after peri-implantation and suffer peri-implantation lethality (Luo et al. 2006). ATF6α/β double knockout causes embryonic lethality starting at embryonic day 8.5 since the transcriptional induction of ER chaperones in response to ER stress is also mediated by ATF6α (Ishikawa et al. 2013). In addition, GRP78 is also involved in the regulation of trophoblastic invasion by Interleukin (IL) II (Sonderegger et al. 2011) and/or interacting with p53 and regulating its stabilization (Arnaudeau et al. 2009). These results indicate that GRP78 is very important for ICM development and the embryonic growth.

Furthermore, GRP78 is also strongly expressed in the heart, neural tube, gut endoderm, somites, and surface ectoderm of mouse embryos during early organogenesis (Barnes and Smoak 2000). Since GRP78 is constitutively expressed as early as the two-cell stage and persists through the blastocyst stage, even early organogenesis stage, GRP78 as a resident lumen protein may be useful for the efficient expression of heterologous genes and meet the increase in protein trafficking activities via GRP78 promoter/enhancer (Kim et al. 1990). It is well known that glucose is required for normal embryonic growth and development. Although GRP78 is induced by the glucose deprivation, its expression in embryonic hearts is elevated by hypoglycemia in a dose-dependent fashion (Barnes et al. 1999). Moreover, GRP78 expression is also induced by fresh glucose-containing medium in mouse embryo cells (Patierno et al. 1987). The most likely explanation is hypoglycemia affects N-linked glycosylation reactions and induces the accumulation of under-glycosylated proteins in the ER. Although the function of GRP78 is currently unknown, its relationship to glucose metabolism during early organogenesis suggests that GRP78 expression may be necessary.

It has been reported that mammalian early embryos are vulnerable to a variety of cellular stresses in vitro environment (Lane and Gardner 2005). These stresses, such as temperature, culture media, changed pH, aberrant gas phases, and even visible light, can reduce embryo viability by altering gene expression, epigenetic mechanisms, metabolism, apoptosis (Abraham et al. 2012). ART (assisted reproductive technologies) is frequently used as a reproduction technique in clinic and livestock

breeding. However, the successful rate is still low. It has been shown that many abnormalities, such as fetal oversize and an increase in the incidence of hydramnios, are present in ruminant species following the culture of preimplantation embryos in vitro (Young et al. 1998). It has been reported that stress-responsive genes, such as CHOP, are expressed from the eight-cell stage onward and expressed at a constant level throughout blastocyst stage when mouse embryos are cultured in KSOM medium (Fleming et al. 1997). In addition, CHOP expression is significantly upregulated in both bovine (Fontanier-Razzaq et al. 2001) and mouse (Fontanier-Razzaq et al. 1999) embryos in response to genotoxic stress: methyl methane-sulphonate (MMS) and sodium arsenite. Both MMS and sodium arsenite are general DNA-damaging agents and are the extreme examples of the types of stress encountered by the embryo in culture. And the CHOP-mediated suppression of the Bcl-2 family proteins expression is modulated by GRP78 synthesis inhibitor momitoxin (VT), which alleviates ERS response and corresponds to enhanced cell survival (Huang et al. 2011). This evidence support the hypothesis that upregulated CHOP protein expression is essential to promote the mitochondria-mediated cell apoptosis via the PERK-eIF2α-ATF4 branch of the UPR signal (Huang et al. 2011).

GRP78 is significantly increased in somatic cell nuclear transfer (SCNT) embryos as compared with that of in vivo-derived blastocysts in bovine, which is closely related with the lower developmental capacity of SCNT embryos following transfer (Canepa et al. 2014). These results indicated that ERS and UPR are initiated during standard treatments of assisted reproductive technologies on embryo including embryo collection, culture, SCNT, and cryopreservation. Moreover, it has been reported that Grp78 is also closely related with normal palate development, cleft palate, forelimb, and limb development in mouse embryo (Zhu et al. 2012, 2013; Yan et al. 2015). Hao's study finds that the abnormal ER structure and accumulated GRP78 in embryo is tightly correlated with the embryo death in the DDK syndrome. The latter is the polar-lethal embryonic death that occurs at the morula-blastocyst transition. The cell apoptosis and the degeneration of embryo may be activated by the elevated ATF4. The association between death of DDK syndrome embryos and induction of the UPR indicate that GRP78 acts as a mediator to initiate the apoptotic cascade when ERS conditions persist (Hao et al. 2009). These results further indicate that GRP78 is closely related to normal and abnormal embryonic development as a chaperone of morphologic development of cells and organisms.

In summary, GRP78 is minimally detected in zygotes and constitutively expressed as early as the two-cell stage. And then its expression persists through the blastocyst stage and even early organogenesis stage. During the embryonic growth and early organogenesis, the extensive new protein syntheses are necessary to maintain these processes. GRP78 may act as central regulator to promote proper protein folding in the lumen of the endoplasmic reticulum (ER). These actions are very essential to maintain the ER homeostasis by UPR, which are vital for normal development of embryo. Therefore, understanding the function and molecular mechanisms of GRP78 underpinning the developmental competence of embryo is significant to the improvement of female reproduction.

2.5 *Effect of Grp78 in Placenta*

The placenta is critical for sustaining the growth of the fetus during pregnancy by facilitating nutrition and gas exchange as well as waste product disposal. Malfunction of placenta causes intrauterine fetal growth retardation, fetal death, and birth defects (Watson and Cross 2005). Increasing evidence demonstrated that ERS are involved in placenta development and functions (Yung et al. 2008; Wang et al. 2012; Fu et al. 2015). Placental ERS is relevant to many pregnancy disorders, including small-for-gestational age at high altitude, intrauterine growth restriction (IUGR), early-onset preeclampsia (PE), and gestational diabetes (Yung et al. 2008, 2012). Excessive and exogenous ER stress may induce functional abnormalities in the placenta and induces low birth weight, which is, in partially, mediated by the changed Glut-1/3 expression (Kawakami et al. 2014).

It is well known that three signaling branches: PERK-pEIF2a, ATF6, and IRE1-XBP1, are involved in the ERS-UPR cascade (Kohno 2007; Lian et al. 2011). According to the earlier report, maternal CdCl2 exposure during pregnancy induces ER stress in placenta and increased the incidence of external malformations in fetuses. These processes are mediated by the PERK signaling branch since CdCl2 significantly increased the level of phosphorylated eIF2α, a downstream target of the PERK pathway in placenta of mice. Moreover, the expression of another two targets of PERK signaling, ATF4 and CHOP, is also increased by CdCl2. However, the IRE1 signaling is not activated (Wang et al. 2012). In addition, nicotine exposure during pregnancy augments placental ER stress, UPR activation, and impairs placental function by acting through its receptor, which may be mediated by the elevated Grp78, phosphorylated eIF2α, Atf4, and CHOP in the placenta (Repo et al. 2014; Wong et al. 2015, 2016).

A recent report demonstrated that activated PERK-peIF2α and ATF6 signaling are associated with decreased cellular proliferation and the impaired placental growth in mothers delivering low birth weight neonates (Lian et al. 2011). The PERK signaling pathway is also involved in PE. PE is a pregnancy-specific disease, which may cause clinical morbidity and mortality in pregnant women and prenatal infants. The early-onset PE is predominantly due to placental pathology (Redman and Sargent 2005; Kim et al. 2007). The lower weight of placenta in PE is closely related with the ERS-induced apoptosis since the PERK signaling pathway markers, including GRP78, PERK, eIF2α, ATF4, CHOP, and caspase 12, are markedly elevated (Yung et al. 2008; Fu et al. 2015). Moreover, the GRP78 expression is higher in early-onset PE than in late-onset PE (Loset et al. 2011; Yung et al. 2014). These results indicate that the placental stress may contribute to the pathophysiology of early-onset preeclampsia.

Both the PERK-pEIF2α and ATF6 signaling branches are activated in decidua basalis tissue from the placenta, and increased XBP1(U) (unspliced Xbox-binding protein) and ATF6 protein are correlated with the reduced placental weight (Lian

et al. 2011). Generally, XBP1 mediates the regulation of the UPR target gene by IRE1α. IRE1α is widely expressed in placenta and plays critical roles in the female reproduction. The inactivation of IRE1α causes widespread developmental defects and leads to embryonic death after 12.5 days of gestation in mice (Zhang et al. 2005). The embryonic lethality is closely related to the functional defects of IRE1α, which is investigated by the IRE1α$^{-/-}$ placental cells. Meanwhile, GRP78 is expressed at high level in an IRE1α-dependent manner. The regulation is accompanied by the active form of the PERK-peIF2a and ATF6α proteins (Iwawaki et al. 2009). However, the expression levels of the ATF6 and Ire1-XBP-1 signaling pathway in the placentas of PE is not consistent (Yung et al. 2014; Fu et al. 2015). Recent report shows that GRP78 is significantly higher in labored placentas (Veerbeek et al. 2015), which is possibly related with the oxidative stress induced by labor (Yung et al. 2014). Further investigations are still needed to uncover the specific functions and mechanisms of GRP78 on placenta.

The above results indicate that physiological intercommunications between placental and the ERS response are essential to the female reproduction. Prolonged ERS alters placental morphology, function, and impairs the fetal growth. And ERS cascades play important roles in pathological conditions. Moreover, GRP78 as a mediator is essential to initiate the three signaling branches: PERK-pEIF2a, ATF6, and IRE1- XBP1. Further research on placental health during pregnancy are necessary to provide novel insight into the mechanisms underlying ERS effects on placental function, and intervention strategies are urgently required to improve the fertility.

3 Conclusion

Taken together, the ER acts as a crucial command center by participating in enormous molecular and cellular activities and affects female reproduction. GRP78 is a key protein in ERS signaling and expressed in the follicle, CL, oviduct, uterus, embryo, and placenta (Table 1). Its expression depends on the female reproductive conditions and the stage of ER stress and UPR. Evidence is accumulating that GRP78 is regulating a wide variety of physiological effects that are essential to the female reproductive functions. GRP78 is a multifunctional protein, which is also involved in many pathological progresses. Further studies are necessary to investigate the in-depth and comprehensive physiology processes and mechanism of GRP78 through transgenic, gene knockout, and knock-in models. In addition, better understanding of the regulatory mechanism of GRP78 in female reproduction can provide further insight about reproductive cell survival and apoptosis, even the infertility. Thus, GRP78 as an ER chaperone may also have a significant impact on the prognostic biomarkers and the treatment of various reproductive diseases, including embryonic lethality, IUGR, PE.

Table 1 Expression of GRP78 and influencing factors

Tissue and cell	GRP78 expression	Effect of factors on GRP78 expression	Reference
Ovary	**Rat:** Stronger staining in Granulosa cell than other cells (theca cell and oocyte) in rat	eCG — hCG ↑	Kogure et al. (2013)
	Expressed in CL	Functional stage of CL ↑ Regression stage of CL ↓ PGF2α ↑ TUDCA ↓	Yang et al. (2015b)
	Mouse: Less staining in the mural granulosa cells of healthy follicle in mouse. No staining in the apoptotic granulosa cells	Tm ↑ Tg ↑	Yang et al. (2013)
	Expressed in COCs	High lipid in follicular fluid ↑	Yang et al. (2012)
	Expressed in CL	Functional stage of CL ↑ Regression stage of CL ↓ Tm ↑	Park et al. (2014)
	Goat: Positive staining in mural granulosa cells and no staining in the theca cells of healthy follicle in goat. GRP78 expression increased with follicular atresia.	Tm ↑ Serum-free ↑	Lin et al. (2012b)
	Cow: Detected in oocyte	FSH (160 mg) and hCG (1500 IU) ↑ GnRH agonist + FSH (320 mg) and hCG (1500 IU) —	Cree et al. (2015)
	Expressed in CL	Functional stage of CL ↑ Regression stage of CL ↓	Park et al. (2013a)

(continued)

Table 1 (continued)

Tissue and cell	GRP78 expression	Effect of factors on GRP78 expression	Reference
Oviduct	**Mouse:** Epithelial cells	Depends on the estrous cycle	Lin et al. (2012a, 2014)
	Human: Epithelial cells Stromal cells Oviductal fluids		Lachance et al. (2007) Marin-Briggiler et al. (2010)
Uterus	**Rat:** Endometrial epithelial cells	Intermediate E_2 ↑ P_4 —	Simmons and Kennedy (2000)
	Mouse: Endometrial epithelial cells	Depends on the estrous cycle and pregnancy stage Intermediate E_2 ↑ Embryo ↑	Lin et al. (2012a, 2014)
	Human: Endometrial epithelial cells Endometrial stromal cells (ESC)	Depends on the menstrual cycle OS ↑	Lachance et al. (2007) Guzel et al. (2011) Gao et al. (2012)
Embryo	**Mouse:** All stage of preimplantation Early organogenesis stage (heart, neural tube, gut endoderm, somites, surface ectoderm, palate, and limb et al.)	Hypoglycemia ↑ Glucose ↑	Abraham et al. (2012), Kim et al. (1990), Barnes and Smoak (2000), Barnes et al. (1999), Patierno et al. (1987), Zhu et al. (2012, 2013), Yan et al. 2015
	Cow:	SCNT embryo ↑	Canepa et al. (2014)
Placenta	**Rat:** Placental trophoblast cells	Nicotine ↑	Wong et al. (2015, 2016)
	Human: Placental trophoblastic cells	Nicotine ↑ PE ↑ Labour ↑	Repo et al. (2014), Fu et al. (2015), Loset et al. (2011), Yung et al., (2008, 2014), Veerbeek et al. (2015)

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