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REVIEW

Heat stress response of male germ cells

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Abstract The vast majority of mammalian testes are located outside the body cavity for proper thermoregulation. Heat has an adverse effect on mammalian spermatogenesis and eventually leads to sub- or infertility. Recent studies have provided insights into the molecular response of male germ cells to high temperatures. Here, we review the effects of heat on male germ cells and discuss the mechanisms underlying germ cell loss and impairment. We also discuss the role of translational control in male germ cells as a potential protective mechanism against heat-induced germ cell apoptosis.

Keywords Hyperthermia · Spermatogenesis · Infertility · Heat stress · Germ cell apoptosis · DNA damage · Stress granules

Introduction

Spermatogenesis is a highly coordinated process entailing cell division and differentiation for the production of haploid male germ cells from diploid progenitor cells. Mammalian spermatogenesis can be divided into pre-meiotic, meiotic, and post-meiotic phases. In the pre-meiotic phase, diploid spermatogonia proliferate mitotically and differentiate into spermatocytes. A primary spermatocyte produces four haploid round spermatids through two meiotic divisions. In the post-meiotic phase of spermiogenesis, round spermatids undergo morphological changes leading to elongated spermatids and mature spermatozoa [1, 2]. This process takes ~35 days in mice and ~75 days in humans and continues throughout life, ensuring a continuous supply of sperm in males [3].

In most mammals, the testes where spermatogenesis takes place are located in the scrotum, outside the body cavity. If the testis fails to descend into the scrotum during development, it is exposed to an elevated temperature and loses germ cells [4–6]. The scrotum is generally 2–7 °C cooler than the core body temperature, and the temperature of the testis is regulated by a heat-exchange system [7–12]. Although the testes of some mammals, including elephants, edentates, and cetaceans, are located within the abdomen, the testes in these animals appear to be cooled by special blood vessels, at least in dolphins and seals [13–16]. Thus, tight thermoregulation of the testis is essential for spermatogenesis, but it remains unclear why most mammals have evolved to maintain their testes at low temperatures [17, 18].

A number of studies conducted over the past several decades have documented the adverse effects of heat on spermatogenesis in diverse mammal species [19–28]. Recent studies have provided new insights into the underlying molecular and cellular mechanisms. In this review, we focus on the effect of heat on male germ cells and the molecular mechanisms involved in this process. We further highlight recent advances in our understanding of the heat stress response of male germ cells. Experimental techniques and physiological outcomes of heat stress on mammalian testes have been thoroughly reviewed elsewhere [17, 29].

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Effect of heat stress on mammalian male germ cells

Germ cell apoptosis

The most significant consequence of the heat stress on the testis is the loss of germ cells via apoptosis. Experimental cryptorchidism, in which one or both testes are surgically exposed to abdominal temperatures, induces DNA fragmentation in germ cells [30–32]. A short exposure of the testes to heat by immersing the scrotum in a hot water bath at 43 °C for 15–20 min also results in germ cell apoptosis [33, 34]. In addition, isolated male germ cells from immature rats undergo apoptosis when maintained at 43 °C for 1 h [35]. The tumor suppressor p53 is a potential inducer of germ cell apoptosis in response to heat. Testicular p53 level is elevated after heat stress and is associated with germ cell loss [36–38]. When cryptorchidism was induced in p53 knockout mice, male germ cell death was delayed but still occurred, suggesting that germ cell apoptosis is mediated by both p53-dependent and p53-independent pathways [39]. Fas may be responsible for the p53-independent phase of germ cell apoptosis [40]. However, subsequent studies have shown that apoptosis is not blocked in *gld* (Fas ligand-defective) and lpr^{cg} (Fas-defective) mice, suggesting that the Fas signaling system is not necessary for heat-induced germ cell apoptosis [41–43].

Recent studies have revealed molecular details of germ cell apoptosis triggered by heat stress. A single instance of scrotal heat stress induces a number of pro-apoptotic processes in male germ cells, such as: relocation of pro-apoptotic Bax (Bcl-2-associated x protein) into nuclear or perinuclear regions; upregulation and phosphorylation of anti-apoptotic Bcl-2 (B cell leukemia/lymphoma 2); cytosolic translocation of cytochrome *c* and DIABLO (direct inhibitor of apoptosis-binding protein with low pI); activation of the initiator caspase 9 and the executioner caspases 3, 6 and 7; and cleavage of PARP (poly(ADP-ribose) polymerase) [41–45]. Q-VD-OPH and minocycline, inhibitors of caspases and cytochrome *c* release, respectively, attenuate heat-induced germ cell apoptosis [46, 47]. Nitric oxide synthase (NOS), p38 mitogen-activated protein kinase (MAPK), and caspase 2 were identified as potential upstream signals of this apoptotic pathway. Studies employing genetic ablation and overexpression of NOS as well as treatment with a NOS inhibitor revealed that NOS is involved in male germ cell apoptosis under heat stress [48–50]. Furthermore, p38 MAPK appears to act upstream of NOS because inhibition of p38 MAPK suppresses NOS induction and cytochrome *c* release and eventually confers resistance to germ cell apoptosis in heat stress conditions [45]. Caspase 2 activation is considered to be upstream of the p38 MAPK pathway because a specific inhibitor of caspase 2 prevents heat-induced activation of p38 and NOS [51]. Taken together, these results suggest that the mitochondria-dependent apoptotic pathway, triggered by activation of upstream signals such as p38 MAPK, is critical for heat-induced germ cell death.

Germ cell apoptosis in response to heat stress occurs in a developmental stage-specific manner. A spatio-temporal rhythm of mammalian spermatogenesis creates a cycle of seminiferous tubules in which certain types of male germ cells are associated with specific developmental stages [52]. In adult rats, for example, there are 14 tubular stages, and germ cell apoptosis is prominent in early (I–IV) and late (XII–XIV) stages after heat treatment at 43 °C for 15 min. The most affected germ cell types are the pachytene and diplotene spermatocytes and the early round spermatids [33]. Similar results have been observed in experimental cryptorchidism of mice and rats [30, 32, 53]. Thus, it is generally accepted that pachytene and diplotene spermatocytes and early spermatids are the most susceptible to heat [17]. It is not known why only certain types of male germ cells are selectively affected by heat. It has been proposed that intratesticular testosterone protects germ cells at the relevant stages (VII–VIII) against heat-induced germ cell apoptosis [33]. A recent study provided evidence for another possibility: stress granules are formed in spermatocytes and spermatogonia after heat stress and confer resistance to apoptosis by suppressing the p38 MAPK pathway [54].

The intensity of heat stress determines the timing of germ cell apoptosis. In mice, for example, the number of apoptotic germ cells begins to increase 6–7 days after experimental cryptorchidism (i.e., exposure to abdominal temperatures) [32], whereas apoptotic germ cells are first observed 8 h after exposure at 43 °C for 20 min [34]. In contrast, a short exposure of the testes to lower temperatures of 39–40 °C has no obvious effect on germ cell death [34]. Thus, male germ cells seem to have a threshold of time–temperature dosage for apoptosis [18, 55]. Consistently, no apoptotic germ cells appear after a 10-min exposure of rat testes at 43 °C, but apoptotic germ cells appear within a few days after a 15-min exposure [20]. Apoptosis is further intensified by increasing the exposure time up to 30 min [20]. Fertility has been adversely affected by repeated exposure of testes to heat, albeit normal after a single exposure [56], suggesting the requirement of a time–temperature dosage for germ cell apoptosis.

Sperm DNA damage

Heat-induced sub- or infertility in males is largely attributed to a reduction in the sperm count via germ cell apoptosis, but there is a strong line of evidence that the

sperm DNA integrity is also affected by heat. Sperm DNA damage, as determined by comet or sperm chromatin structure assays, is detected in mature spermatozoa a few days as well as weeks after the heat stress [57–60]. This result indicates that the chromatin integrity of male germ cells at the epididymal sperm stage and of developing spermatocytes is affected by heat. Indeed, heat stress induces defects in DNA synapsis and DNA strand breaks in pachytene spermatocytes [60]. A failure in the X and Y chromosome pairing has also been noted in primary spermatocytes [61, 62]. These damaged spermatocytes may avoid heat-induced apoptosis and develop to mature spermatozoa with defects in their chromatin integrity.

It is well known that oxidative stress, which is caused by the excessive generation of reactive oxygen species (ROS), can also induce DNA damage in spermatozoa [63–66]. Heat stress is implicated in the induction of oxidative stress within the testis [35, 67–69]. Interestingly, several antioxidants have attenuated heat-induced germ cell apoptosis [35, 70]. Furthermore, male germ cells from mice lacking a superoxide dismutase are highly sensitive to heat stress [71]. These studies have not directly evaluated sperm DNA damage, but the treatments would be expected to influence the levels of DNA damage in germ cells. It is also considered that sperm DNA damage can be caused by impaired DNA repair system in spermatocytes [60, 72]. A microarray-based study has shown that some genes for DNA repair and cellular antioxidants are down-regulated in the heat-stressed testis [34]. Taken together, these results suggest that heat stress induces DNA damage in germ cells by increasing ROS and suppressing gene expression associated with defense mechanisms against DNA damage.

It has long been noted that fertilization and normal embryonic development are affected by paternal heat stress. When embryos are sired by heated males, their mortality rate increases [25, 34, 73–75] and their growth is frequently retarded [60, 76–79]. When in vitro fertilization is performed with the sperm of males exposed to heat, the sperm penetration rate, fertilization rate, and pronuclear formation rate decrease [76, 79–81], and defects in early embryonic development are observed [60, 82]. These defects may reflect harmful effects of sperm DNA damage on diverse aspects of fertility, though these studies did not explicitly show a link between DNA damage and fertility outcomes. Future studies should be conducted to determine whether sperm DNA damage is responsible for these deleterious phenotypes.

Other effects

The male germ cell is not the only cell type affected by heat; testicular somatic cells, specifically, Sertoli and Leydig cells, also respond to heat stress. Sertoli and Leydig cells, whose main functions are to support germ cells and regulate steroidogenesis, respectively, provide germ cells with the proper environment for development. Any misregulation of their functions might influence male germ cell development. A decrease in the production of testicular androgen binding protein after experimental cryptorchidism suggests that heat has an adverse effect on Sertoli cells [83, 84]. Recent work has revealed that the expression of some molecules involved in Sertoli cell function is affected by hyperthermic conditions [85–89]. In contrast, it was believed that Leydig cells are not affected by heat [29]. However, degenerative morphology of Leydig cells was observed after six consecutive daily exposures of rat testes to 43 °C for 30 min [90, 91]. In addition, there is evidence that HIF1A (hypoxia-inducible factor 1 alpha) and HMOX1 (heme oxygenase 1) are up-regulated in Leydig cells, suggesting that heat induces hypoxia and oxidative stress in these cells [69, 92]. Therefore, it is possible that heat may indirectly harm germ cells by modifying somatic cell functions.

Elevated temperature has also been reported to increase the rate of spermatocyte differentiation and the progression of spermatogenesis [93, 94]. The reduced cycle of spermatogenesis may lead to the disruption of spermatogenic cells. However, it is still unknown how heat modulates the timing of the spermatogenic cycle.

Testis is known to recover from the heat damage with time. However, the scar can be too deep to be fully recovered. Testis loses its weight after a single heat exposure and then gains its weight about 40 days later, but the weight does not return to normal level even 60 or more days later [95, 96]. In a study with an extended observation, testis weight was recovered up to 70 % of control at 97 days after heating, but there was a second fall to 50 % at 182 days later [97]. The initial decline in testis weight after heat stress is largely attributed to the apoptosis of heat-susceptible germ cells discussed above. However, the existence of a second fall in testis weight after heating suggests the possibility that heat also affects the differentiation and development of spermatogonia by unknown mechanisms [97, 98].

Gene expression changes in male germ cells after heat stress

Hyperthermic effects on male germ cells lead to changes in gene expression, posttranslational modification, and protein localization. Several studies on such changes have focused on identifying the underlying molecular mechanisms of thermal effects on spermatogenesis. In this section, we summarize the molecular changes so far examined, which have been confirmed in male germ cells with defined

regimes of heat stress. Although DNA microarray [34, 68] and proteomic analysis [99, 100] of heat-treated testes can, in an unbiased manner, provide valuable information for our understanding of how male germ cells respond to elevated temperature, we do not address these data in our discussion to avoid confounding effects originating from the testicular somatic cells.

Genes implicated in apoptosis

Apoptosis is a crucial cellular response for the maintenance and homeostasis of organisms, and, as such, its pathway is highly conserved in a metazoan lineage [101]. As we have discussed, many conserved factors of the intrinsic death pathway are involved in heat-induced germ cell apoptosis. Accordingly, heat stress triggers molecular changes that reflect the ongoing process of apoptosis in male germ cells (Table 1). Many factors that positively induce apoptosis are up-regulated [37, 41, 44, 45, 48, 51, 102–108], while some are down-regulated [37, 102]. Other molecular changes include protein translocation [41, 43–45, 47, 51, 105], modifications such as acetylation and phosphorylation [45, 51, 102, 105, 107, 109], and cleavage [41, 43, 47, 51, 69], all of which are prominent features of apoptosis.

Heat shock protein genes

In response to heat stress, heat shock proteins (HSPs) play protective roles in preventing the nonspecific aggregation and thermal denaturation of cellular proteins. Protective mechanisms of HSPs, such as molecular chaperones, are conserved among all living cells [110]. Due to their vital function in heat-protective mechanisms, HSPs have attracted much attention in the field of male germ cell research. Two types of HSPs are identified in male germ cells: constitutively expressed HSPs and heat-inducible HSPs [111]. The first group of HSPs, which includes Hsp70-2, is not heat-inducible and seems to be developmentally regulated to function in normal spermatogenesis [112, 113]. The latter group includes Hsp70-1 and Hsp70-3, whose expression is induced in response to various thermal conditions [34, 114–118] (Table 1).

In general, heat-inducible HSPs are activated by heat shock transcription factors (HSFs). HSF1 undergoes protein modification and is thus activated in heat-stressed spermatocytes [115]. Considering the central role of HSPs in cellular thermotolerance, one would expect that the heat-inducible HSPs triggered by HSF1 protect against the adverse effects of heat. Currently, however, there is no direct evidence to support this view. Rather, studies have shown that HSF1 promotes germ cell death independent of the activation of HSP genes [104, 119, 120] and that constitutive expression of the heat-inducible Hsp70-1 in spermatocytes protects against neither heat-induced nor HSF1-induced apoptosis [121, 122]. Although the opposite role of HSF1 as a cell survival factor in spermatogonia has been suggested, this is also independent of the activation of HSP genes [120]. A recent study has identified numerous meiotic genes to be regulated by HSF1 in oocyte maturation [123]. Thus, it is possible that the HSF1–HSPs pathway is differently regulated in somatic and male germ cells, but this issue remains to be further investigated.

Other genes

The expression of other genes in male germ cells also appears to be affected by heat stress. A few genes that are possibly related to apoptotic processes are up-regulated [109, 124, 125], whereas genes for DNA repair and cell cycle regulation are down-regulated after heat stress [126–129]. It is possible that the down-regulation of DNA repair genes impairs the chromatin integrity of sperm in heat stress conditions. However, in most cases, it is not clear whether the down-regulation of gene expression contributes to germ cell death or survival or whether the decreased expression merely reflects impaired function due to cellular damage.

Translational control in male germ cells after heat stress

The regulation of translation plays a pivotal role in cellular response to unfavorable, stressful conditions. Translational regulation allows the stressed cells to respond immediately and selectively via rapid changes in protein levels [130]. Several lines of investigation have revealed that male germ cells exposed to heat stress undergo changes in gene expression at the level of translation. It has been reported that heat reduces the incorporation of amino acids into proteins and polysome formation [131–133]. The decline of protein synthesis is linked to a limited formation of the translation initiation complex [134]. A recent study provides a possible explanation of the molecular and cellular mechanisms responsible for these translational changes: a short exposure of mouse testes at 37 °C for 20 min triggers an immediate phosphorylation of eIF2α, the α subunit of eukaryotic translation initiation factor 2, and the stage-specific formation of stress granules (SGs) in male germ cells [54]. The implications of this finding will be discussed with respect to germ cell survival and a lowered set point for heat stress response.

eIF2α phosphorylation

Various stresses (e.g., nutritional deprivation, viral infection, UV irradiation, hypoxia, oxidative stress, and heat)

Table 1 Molecular changes in male germ cells after heat stress

Gene	Name	Function	Change	Level	Heat stress	References
Apoptosis-related genes						
Fas	TNF receptor superfamily member 6	Induction of apoptosis	Up	mRNA/protein	EC, LH	[40, 104]
			No[a]	Protein	LH	[41, 105]
Htra2	HtrA serine peptidase 2	Proteolysis	Up	mRNA/protein	EC	[103]
Nos2	Nitric oxide synthase 2	Oxidation–reduction process	Up	Protein	LH	[45, 48, 51, 106]
p21	Cyclin-dependent kinase inhibitor 1A	DNA damage response	Up	mRNA/protein	EC, IH	[102, 107]
p53	Transformation related protein 53	DNA damage response	Up	mRNA/protein	EC, IH, LH	[37, 102, 107]
			Acetylation	Protein	IH	[107]
Phlda1	Pleckstrin homology-like domain, family A, member 1	Induction of apoptosis, FasL biosynthetic process	Up	mRNA/protein	EC, LH	[104, 108]
Bcl-2	B cell leukemia/lymphoma 2	Anti-apoptosis	Up	Protein	LH	[41, 44, 105]
			Phosphorylation	Protein	LH	[45, 105]
Nr2c1	Nuclear receptor subfamily 2, group C, member 1	Regulation of transcription	Down	mRNA	EC	[102]
			No[a]	mRNA	LH	[37]
Nr2c2	Nuclear receptor subfamily 2, group C, member 2	Regulation of transcription	Down	mRNA	EC, LH	[37, 102]
Nr4a1	Nuclear receptor subfamily 4, group A, member 1	Regulation of transcription	Down	mRNA	LH	[37]
			No[a]	mRNA	EC	[102]
Bax	Bcl-2-associated X protein	Apoptotic mitochondrial changes	Translocation	Protein	LH	[41, 43, 44, 105]
Cyc	Cytochrome *c*	Apoptotic process, electron carrier activity	Translocation	Protein	LH	[41, 43, 45, 51, 105]
Diablo	Direct inhibitor of apoptosis-binding protein with low pI	Induction of apoptosis	Translocation	Protein	LH	[43, 47, 105]
Erk	Mitogen-activated protein kinase 1/3	Stress-activated MAPK cascade	Phosphorylation	Protein	LH	[45, 105]
p38	Mitogen-activated protein kinase 14	Stress-activated MAPK cascade	Phosphorylation	Protein	LH	[45, 51, 109]
Rb	Retinoblastoma 1	Negative regulation of cell cycle	Hypo-phosphorylation	Protein	EC	[102]
Casp2	Caspase 2	Induction of apoptosis	Cleavage	Protein	LH	[51]
Casp3	Caspase 3	Induction of apoptosis	Cleavage	Protein	LH	[41, 43, 47, 51, 69]
Casp9	Caspase 9	Induction of apoptosis	Cleavage	Protein	LH	[41, 43, 47, 51]
Parp1	Poly (ADP-ribose) polymerase family, member 1	DNA repair	Cleavage	Protein	LH	[41, 47]
Heat shock protein-related genes						
Hsp60 (*Hspd1*)	Heat shock protein 1 (chaperonin)	Response to unfolded protein	Up	mRNA/protein	LH	[117]
Hsp70-1 (*Hspa1b*)	Heat shock protein 1B	Response to heat, anti-apoptosis	Up[b]	mRNA/protein	LH	[34]
Hsp70-3 (*Hspa1a*)	Heat shock protein 1A	Response to heat, DNA repair	Up	mRNA/protein	IH, LH	[34, 114, 115, 118]

Table 1 continued

Gene	Name	Function	Change	Level	Heat stress	References
Hsp105 (*Hsph1*)	Heat shock 105 kDa/110 kDa protein 1	Response to unfolded protein	Up[b]	Protein	IH	[116]
			Translocation	Protein	EC	[116]
			Down[a]	mRNA/protein	LH	[117]
Hsp70-2 (*Hspa2*)	Heat shock protein 2	Male meiosis, response to stress	Down	mRNA/protein	EC, LH	[27, 118, 178]
			No[a]	mRNA/protein	IH, LH	[120, 179]
Hsc70t (*Hspa1 l*)	Heat shock protein 1-like	Response to stress	Down	mRNA	LH	[118]
Hsf1	Heat shock factor 1	Response to heat, regulation of transcription	Down	mRNA/protein	EC	[108]
			DNA binding activity	Protein	IH, LH, EC	[115, 119, 120]
Hsf2	Heat shock factor 2	Regulation of transcription	Down	mRNA/protein	EC	[108]
Other genes						
Capn2	Calpain 2	Proteolysis	Up	mRNA	LH	[109]
Ptgs2	Prostaglandin-endoperoxide synthase 2	Oxidation–reduction process	Up	mRNA/protein	EC	[125]
Ucp2	Uncoupling protein 2	Response to superoxide	Up	Protein	LH	[124]
Capn11	Calpain 11	Proteolysis	Down	mRNA	LH	[109]
Ccnb1	Cyclin B1	Regulation of cell cycle	Down	Protein	EC	[127]
Cdk1	Cyclin-dependent kinase 1	Regulation of cell cycle	Down	Protein	EC, LH	[127, 129]
Cirp	Cold inducible RNA binding protein	Stress response, translational regulation	Down	mRNA/protein	IH, LH, EC	[59, 180]
Csda	Cold shock domain protein A	Regulation of transcription	Down	mRNA/protein	IH, LH, EC	[181]
Lig3	DNA ligase III	DNA repair	Down	Protein	EC	[128]
Mta1	Metastasis associated 1	Regulation of transcription	Down	mRNA/protein	IH, LH, EC	[107, 182]
Pde4a	Phosphodiesterase 4A, cAMP specific	Metabolic process	Down	Protein	EC	[183]
Polb	DNA polymerase beta	DNA repair	Down	mRNA	EC	[126]
Prm2	Protamine 2	Chromosome condensation	Down	mRNA	EC, LH	[181]
Eif2s1	Eukaryotic translation initiation factor 2, subunit 1 alpha	Regulation of translation	Phosphorylation	Protein	LH	[54]

Up up-regulation, *No* no change, *Down* down-regulation, *EC* experimental cryptorchidism, *LH* local heating, *IH* in vitro heating

[a] Conflicting results for the same gene

[b] Other studies suggest that this gene is up-regulated not in germ cells but in somatic cells [104, 120]

are known to trigger eIF2α phosphorylation at residue Ser-51 in eukaryotic cells. Phosphorylation of eIF2α inhibits the catalytic activity of eIF2B, which converts a GDP-bound eIF2 into an active GTP-bound eIF2 and thereby reduces the formation of the eIF2-GTP-Met-tRNA$_i$ ternary complex, leading to the global attenuation of translation initiation [135–137]. Paradoxically, reduction of the ternary complex formation by eIF2α phosphorylation can also elicit selective translation of a few mRNAs involved in stress response. Such selective translation under stress is well understood for two transcriptional activators, GCN4 (general control non-derepressible 4, an activator of amino acid biosynthetic genes) [138] and ATF4 (activating transcription factor 4, an activator of unfolded protein response genes) [139] (for the regulatory mechanisms, see [130, 137]). Taken together, these mechanisms are thought to promote adaptive stress response for cell survival against adverse circumstances. However, some evidence also implicates eIF2α phosphorylation in apoptosis under stress conditions such as proteasome inhibition and hypertonicity [140, 141]. Thus, the effects of eIF2α phosphorylation on cells appear to be dependent on stress conditions. A recent study of eIF2α phosphorylation in heat-stressed testes [54] suggests that male germ cells can be controlled by similar molecular mechanisms. Heat induces eIF2α phosphorylation in male germ cells, and this effect, in turn, might attenuate general translation by reducing the availability of the ternary complex; this effect might also enhance the translation of selective mRNAs implicated in the adaptive stress response. This scenario could explain the previous observations of decreased incorporation of amino acids, reduction of polysomes, and slower formation of the translation initiation complex in heat-stressed spermatogenic cells [131–134]. Consequently, eIF2α phosphorylation may serve as a trigger of cellular survival pathways in male germ cells under heat stress, although its contribution to germ cell apoptosis cannot be excluded.

In the mouse testis, eIF2α can even be phosphorylated at 37 °C, which is similar to the core body temperature, and it has been shown that germ cells are highly responsive to its phosphorylation [54]. This result is interesting because a higher temperature, usually 43–44 °C, is required to induce eIF2α phosphorylation in mammalian somatic cells. A similar, lowered temperature threshold for molecular changes in male germ cells has been noticed in HSF1 activation. It has been shown that HSF1 is even activated at 35–38 °C in male germ cells but is activated at 42 °C in somatic cells [115, 142]; the activity of HSF1 is facilitated by posttranscriptional modifications, such as phosphorylation, at many residues, although how HSF1 senses heat stress is poorly understood [143]. Therefore, to understand how male germ cells have adapted to have a lowered temperature threshold, it would be useful to find the kinases that regulate phosphorylation in response to mild heat stress. In mammalian cells, the phosphorylation of eIF2α is mediated by four different stress-sensing kinases: (1) HRI (heme-regulated inhibitor), which is activated by heme deprivation as well as oxidative, osmotic, and heat stress in erythrocytes [144, 145]; (2) PKR (double-stranded RNA-dependent protein kinase), which is activated by viral infection [146]; (3) PERK (PKR-like ER kinase), which is activated by unfolded proteins in the endoplasmic reticulum (ER stress) [147]; and (4) GCN2 (general control non-derepressible 2), which is responsive to amino acid deficiency and UV irradiation [148, 149]. With a conserved catalytic domain, all four kinases specifically phosphorylate eIF2α on Ser-51 [137]. Thus, it is possible that one or more kinases also control eIF2α phosphorylation in heat-stressed male germ cells. One such candidate may be HRI: it has been shown that HRI is activated in heat-shocked erythroid cells [145], and Hri2p, a HRI-related enzyme, is required for eIF2α phosphorylation by heat shock in the fission yeast, *Schizosaccharomyces pombe* [150]. Alternatively, there may be a novel, testicular version of an eIF2α kinase in male germ cells.

SG formation

In addition to eIF2α phosphorylation, heat stress induces the transient formation of SGs in mouse male germ cells (Fig. 1). SGs, non-membranous cytoplasmic particles that contain untranslated messenger ribonucleoproteins (mRNPs), are transiently accumulated in response to adverse environmental stress [151]. Consistently, the core components of SGs are the stalled pre-initiation 48S complexes including polyadenylated mRNAs, 40S ribosomal subunits, and translation initiation factors such as eIF3, eIF4E, and eIF4G [152, 153]. In addition, proteins that regulate RNA metabolism, such as RNA-binding proteins, RNA helicases and mRNA-editing enzymes, microRNAs, and some signaling molecules have been shown to accumulate in SGs [154]. Because the mRNPs of SGs are in dynamic equilibrium with the polysomes [155], the assembly and disassembly of SGs are predominantly regulated by the status of the translational machinery: stress induces the phosphorylation of eIF2α, which in turn triggers SG assembly by preventing translation initiation, while recovery after non-lethal stress induces rapid disassembly of SGs [152]. SGs are thought to function in the regulation of mRNAs, such as their storage, degradation, or translation reinitiation during stress and recovery conditions [156]. SGs have also been thought to regulate cell survival by recruiting signaling molecules into SGs [157, 158]. In this regard, SGs of male germ cells are likely to play roles in cell survival against heat stress by protecting or regulating translationally dormant mRNAs and by

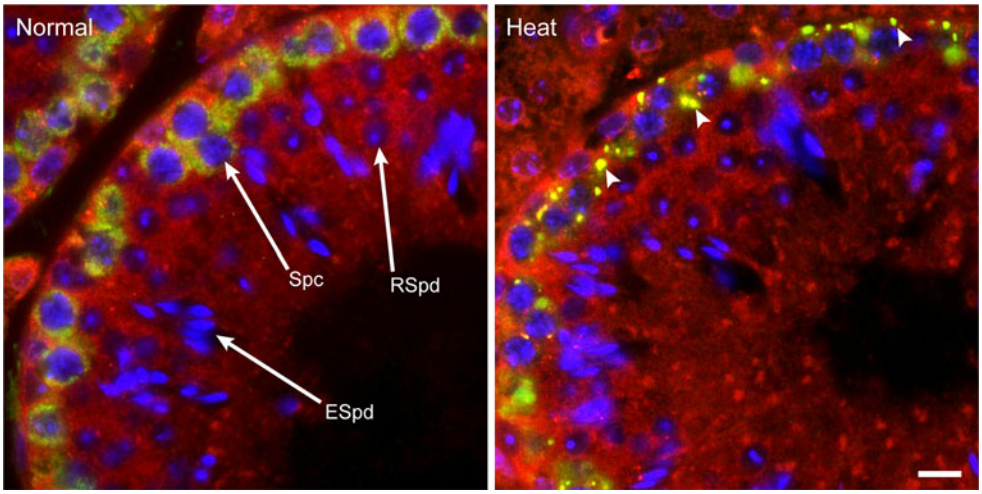

Fig. 1 Formation of stress granules (SGs) in male germ cells. Adult mouse testes of control (*Normal*) and heated at 42 °C for 20 min (*Heat*) were costained for two robust markers of SGs, DAZL (*green*) and eIF3 (*red*). DNA was stained with DAPI (*blue*). The images were taken from the seminiferous epithelial stage IV. *Arrowheads* indicate SGs. Note that SGs were predominantly formed in pachytene spermatocytes. *Spc* spermatocyte; *RSpd* round spermatid; *ESpd* elongated spermatid. *Scale bar* 10 μm

sequestering proteins that regulate signaling pathways involved in apoptosis.

How are SGs assembled in male germ cells? Using a systematic RNAi screen, several factors involved in SG assembly have been identified in cultured cells [159]. In male germ cells, however, we so far have only one such example. It has been shown that DAZL (deleted in azoospermia-like), a germ cell-specific translational regulator, localizes to SGs upon heat stress (Fig. 1) and is required for SG formation in male germ cells. DAZL is thought to act downstream of eIF2α phosphorylation for SG assembly because eIF2α was phosphorylated at a similar level upon heat stress even in the absence of DAZL [54]. *Dazl* belongs to the evolutionarily conserved *DAZ* gene family: in humans, for example, there are four copies of *DAZ* genes on the canonical Y chromosome that are expressed in the testis [160, 161] and two autosomal homologues, *DAZL* and *BOULE* [162, 163]. Deficiency of the *DAZ* gene family members has been shown to lead to infertility in many animals [164–168], indicating their conserved functions in germ cell development. Recent work has also revealed the functional roles of *DAZ* family genes in meiotic initiation and progression and early embryonic development [169–171]. The molecular function of the RNA-binding protein DAZL has been suggested to activate translation and facilitate transport of specific mRNAs [171–175]. Therefore, DAZL seems to contribute to germ cell development by translational regulation of its bound mRNAs in normal conditions as well as by SG formation and, possibly, accompanied translational regulation in heat stress conditions. It is still unknown whether the SG formation activity of DAZL is shared by other DAZ family proteins. This is of interest because BOULE, for example, is highly expressed in later stages of germ cell development than those showing DAZL expression [163, 176]. This issue warrants further investigation.

In male germ cells, SG formation occurs only in selective germ cell types (Fig. 1). For example, DAZL-positive SGs have been found in spermatogonia, preleptotene, and early pachytene spermatocytes, but not in leptotene, zygotene, and late pachytene spermatocytes or in spermatids of all stages [54]. Although the reason SGs are selectively induced in certain cell types remains elusive, this finding suggests a possible link between SG formation and heat-induced germ cell apoptosis with respect to their cell type specificity. SG formation has been shown to inhibit apoptosis by suppressing the p38 MAPK signaling pathway in cultured cells [158], and p38 MAPK is involved in germ cell apoptosis after heat stress [45]. As we have already discussed, heat-induced germ cell apoptosis is stage and cell type specific. Several lines of evidence support the hypothesis that SG formation inhibits germ cell apoptosis in a male germ-line: there is a negative correlation between the cell types that form SGs and those that undergo apoptosis after heat stress, in which SG formation occurs predominantly in early pachytene spermatocytes and apoptosis in late pachytene spermatocytes; a higher rate of apoptosis is induced in the *Dazl* knockout testes that have shown impaired SG formation; RACK1 (receptor for activated protein kinase C), an activator of the stress-responsive p38 MAPK pathway, is sequestered into SGs in wild-type germ cells but not in the *Dazl* knockout cells; and p38 MAPK activation is up-regulated in germ cells that contain SGs [54]. Thus, SGs are likely to prevent the male germ cells that harbor them from undergoing apoptosis after heat stress. To better understand the cell type

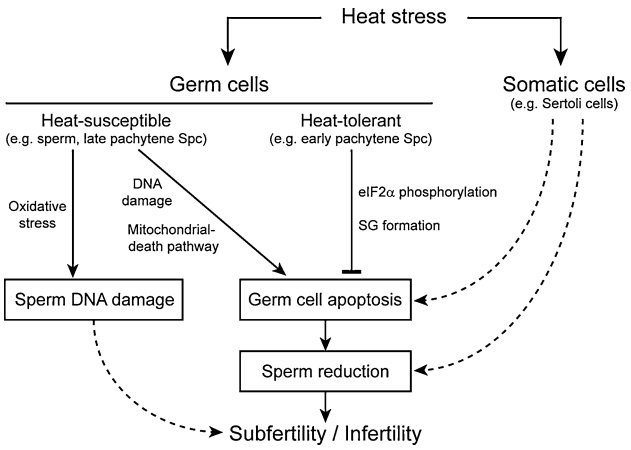

Fig. 2 Model for heat stress response in male germ cells. Heat stress affects germ cells as well as somatic cells, leading to disruption of normal spermatogenesis. The cellular responses differ between different types of germ cells. *Spc* spermatocytes; *SG* stress granule

specificity of SGs, more SG markers should be identified and tested for in the male germ cells that contain SGs. Furthermore, studies that find novel factors involved in SG formation and mutants showing abortive SG formation in their germ cells will help to reveal the relationship between SG formation and germ cell apoptosis.

Conclusion and future studies

Testes contain the spermatogenic cells at all stages of maturation, as well as somatic cells, both of which can affect the overall fertility of an animal exposed to heat stress (Fig. 2). The heat-susceptible germ cells undergo either apoptosis through the mitochondrial death pathway or DNA damage even if they have escaped apoptosis. The sperm in the epididymis is also affected by heat, which leads to sperm DNA damage that might result in subfertility of the affected male. Groups of the heat-resistant germ cells often form SGs and are subjected to translational control in response to heat stress; this control at the translation level is likely to inhibit apoptosis and protect the germ cells transiently from the unfavorable condition. Heat stress on the somatic compartment of the testis induces changes in gene expression that might impair normal spermatogenesis.

Although there has been much progress in understanding the heat stress response of male germ cells, some issues still remain unresolved. Why are some male germ cells susceptible to heat stress, while others often form SGs and are tolerant? Because these germ cells are in specific phases of the meiotic cell cycle, it is possible that heat susceptibility or SG formation is a cell cycle-dependent event. Such a cell cycle dependency has been found in SG formation of cultured cells after UV irradiation [177]. It will be interesting to determine whether both apoptosis and SG formation upon heat stress are dependent on the cell cycle. Another interesting issue is whether the molecular changes in male germ cells after heat stress are causes or effects of the modulation of cellular behaviors. Most of the up-regulated genes seem to participate in germ cell death, or cell survival, while it is still unclear whether the down-regulation of genes is functional or simply lowered due to germ cell loss. Genetic studies for these molecules, in conjunction with further efforts to find new genes implicated in the heat stress response, will provide insight into how complex genetic networks regulate the behavior of male germ cells under heat stress conditions. However, although we increasingly understand the mechanisms behind the heat stress response of the testis, the evolutionary reason behind this sensitivity remains a mystery; female gametogenesis happens happily at 37 °C.

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