Heat-shock protein HSPA4 is required for progression of spermatogenesis

Torsten Held, Amal Z Barakat, Belal A Mohamed, Ilona Paprotta, Andreas Meinhardt¹, Wolfgang Engel and Ibrahim M Adham

Institute of Human Genetics, University of Göttingen, D-37073 Göttingen, Germany and ¹Department of Anatomy and Cell Biology, University of Giessen, D-35378 Giessen, Germany

Correspondence should be addressed to I M Adham; Email: iadham@gwdg.de

T Held and A Z Barakat are contributed equally to this work and should be considered co-first authors

Abstract

Heat-shock protein 110 (HSP110) family members act as nucleotide exchange factors (NEF) of mammalian and yeast HSP70 chaperones during the ATP hydrolysis cycle. In this study, we describe the expression pattern of murine HSPA4, a member of the HSP110 family, during testis development and the consequence of HSPA4 deficiency on male fertility. HSPA4 is ubiquitously expressed in all the examined tissues. During prenatal and postnatal development of gonad, HSPA4 is expressed in both somatic and germ cells; however, expression was much higher in germ cells of prenatal gonads. Analyses of *Hspa4*-deficient mice revealed that all homozygous mice on the hybrid C57BL/6J×129/Sv genetic background were apparently healthy. Although HSPA4 is expressed as early as E13.5 in male gonad, a lack of histological differences between *Hspa4*^{−/−} and control littermates suggests that *Hspa4* deficiency does not impair the gonocytes or their development to spermatogonia. Remarkably, an increased number of the *Hspa4*-deficient males displayed impaired fertility, whereas females were fertile. The total number of spermatozoa and their motility were drastically reduced in infertile *Hspa4*-deficient mice compared with wild-type littermates. The majority of pachytene spermatocytes in the juvenile *Hspa4*^{−/−} mice failed to complete the first meiotic prophase and became apoptotic. Furthermore, down-regulation of transcription levels of genes known to be expressed in spermatocytes at late stages of prophase I and post-meiotic spermatids leads to suggest that the development of most spermatogenic cells is arrested at late stages of meiotic prophase I. These results provide evidence that HSPA4 is required for normal spermatogenesis.

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Introduction

Complexes containing heat-shock proteins (HSPs) represent the major components of molecular chaperones that facilitate the folding and assembly of newly synthesized proteins and the selection of unfolded proteins for degradation in different cellular compartments such as cytosol, endoplasmic reticulum, and mitochondria (Hartl 1996). Based on the molecular weight, HSPs are divided into structurally unrelated HSP110, HSP90, HSP70, HSP60, and HSP27 protein families (Vos *et al.* 2008).

The *HSP110* gene family includes two genes in *Saccharomyces cerevisiae* known as *SSE1* and *SSE2* and four genes in the mammalian genome, namely *HSPA4I/APG1*, *HSPA4/APG2*, *HSPH1/HSP105*, and *HYOU1/GRP175/ORP150*. Except *HYOU1*, which is present in the endoplasmic reticulum, all other members of mammalian and yeast HSP110 are found in the cytosolic compartment (Vos *et al.* 2008). Primary structure of HSP110 proteins is highly related to HSP70 and consists of a nucleotide-binding domain (NBD) and

a peptide-binding domain (PBD) that are connected by a flexible linker region (Mayer & Bukau 2005, Liu & Hendrickson 2007). However, biochemical analyses revealed that HSP110 members are co-chaperones of mammalian and yeast HSP70 chaperones and act as nucleotide exchange factors (NEF) during the ATP hydrolysis cycle (Steel *et al.* 2004, Dragovic *et al.* 2006, Raviol *et al.* 2006, Polier *et al.* 2008, Schuermann *et al.* 2008). Binding of newly synthesized polypeptides to HSP70 chaperone and subsequent release of folded proteins is regulated by a continuous cycle of ATP hydrolysis and exchange of ATP to ADP.

Beside HSP110 proteins, there are other unrelated nucleotide exchange factors of BAG and HSPBiP1 protein families. It is believed that the chaperones containing HSP70, HSP40, and HSP110 proteins represent the major protein folding machine of the eukaryotic cytosol (Polier *et al.* 2008).

Male germ cells undergo a dramatic developmental process, which is precisely controlled at the level of transcription and translation. After colonizing the

primordial germ cells in mouse testis, gonocytes proliferate until day 15.5 and arrest in the G1 of cell cycle (Vergouwen *et al.* 1991, Nagano *et al.* 2000). Shortly after birth, gonocytes resume mitotic activity and develop into type A spermatogonia (McLean *et al.* 2003). Primary spermatocytes undergo meiotic division to produce haploid round spermatids that are subsequently differentiated into mature sperm. Chaperone activities are required to mediate folding of *de novo* synthesized proteins and refolding of misfolded proteins during development of male germ cells (Eddy 1999). Mutation in the *Hspa2* gene (*Hsp70-2*), which is highly expressed in pachytene spermatocytes, leads to arrest the spermatogenesis in meiotic prophase I and the majority of late pachytene spermatocytes are eliminated by apoptosis (Dix *et al.* 1997). Loss of DNAJA1, a co-chaperone of HSP70s in protein folding, results in severe defects of spermatogenesis (Terada *et al.* 2005).

To date, relatively little is known about the expression and the physiological function of HSPA4. In adult mouse, *Hspa4* mRNA is detected in most tissues, with the highest expression in testis, ovary, and spleen (Kaneko *et al.* 1997). *In vitro* studies have shown that the expression of *Hspa4* is not inducible by heat shock (Nonoguchi *et al.* 1999). Interestingly, expression of *Hspa4* is up-regulated in some leukemia and solid tumors (Gotoh *et al.* 2004, Li *et al.* 2010).

To determine the expression pattern of HSPA4, we investigated the expression of HSPA4 in different tissues and during prenatal and postnatal testis development. To examine the function of HSPA4 *in vivo*, we generated *Hspa4*-deficient mice and subsequently determined the effect of HSPA4 deficiency on germ cell development.

Results

HSPA4 is highly expressed in embryonic gonocytes and oögonia

To investigate the expression pattern of HSPA4, northern blot analysis was performed with total RNA from different tissues of adult mice. The 358 bp cDNA probe containing the coding sequence of the C-terminus recognized two *Hspa4* transcripts of 4.8 and 3.2 kb in all studied tissues. Expression levels of the 3.2 kb transcript were relatively higher in testis (Fig. 1A). Analysis of *Hspa4* cDNA sequences in database revealed that a cDNA sequence (GI 60360215; AK 220167) contains two predicted polyadenylation sites that are spanning 1.75 kb region. To confirm that both mRNA isoforms result from alternative splicing of the 3'-untranslated region, an RNA blot was hybridized with the 596 bp cDNA probe containing the sequence between both polyA signals. This probe only detected the 4.8 kb transcript (Fig. 1B). To further confirm these

Figure 1 Expression pattern of HSPA4 in different tissues, fetal and postnatal testes, testes of different mutant mice, and different cell lines. (A) Northern blot with total RNA from different tissues of 3-month-old mice was hybridized with the 358 bp *Hspa4* cDNA probe. The same blot was rehybridized with a β -actin cDNA probe. (B) RNA blot with RNA of adult testis was hybridized with the 596 bp *Hspa4* cDNA probe. (C) Immunoblot of HSPA4 expression in cellular extracts from different tissues of 3-month-old wild-type (WT) and mutant mice (KO) revealed that anti-HSPA4 recognized a 96 kDa protein in all wild-type tissue, but not in *Hspa4*-deficient tissues. (D and E) Expression of HSPA4 during prenatal (D) and postnatal testis development (E) was examined by immunoblotting using total lysates obtained from testes of E13.5, E15.5, and E17.5 embryos, and from P5, P10, P15, P20, P30, and P60 mice. The anti- α -tubulin antibody (α -Tub) was used as a control. (F) Western blot with lysates obtained from testes of wild-type (+/+) and different mutant mice was probed with anti-HSPA4 antibody. (G) Immunoblot of HSPA4 expression in cellular extracts from different cell lines. ESC, embryonic stem cells; SSC, spermatogonial stem cells; HeLa, HeLa cells; 15P-1, Sertoli cells, Hep2G, Hepatoma cells.

results, western blot containing protein extracts of different wild-type and *Hspa4*-deficient tissues was probed with the anti-HSPA4 antibody. Immunoblot analysis revealed that the anti-HSPA4 recognized a 96 kDa protein in all wild-type tissues, but not in *Hspa4*-deficient tissues (Fig. 1C). To evaluate the expression pattern of HSPA4 during testis development, immunoblot analysis was performed with testis extracts obtained from mice at the different stages of prenatal and postnatal development. HSPA4 was detected throughout the prenatal and postnatal development of testis as shown in Fig. 1D and E. Expression levels of the HSPA4 are markedly increased in testis after postnatal day 20 suggesting an increased expression of the HSPA4 in haploid spermatids. We also examined the expression of HSPA4 in testes of mouse mutants. HSPA4 was present in testes of *W/W^v* mutant mice that lack most germ cells as well as in testes of *qk/qk*, *olt/olt*, and *Ins13^{-/-}* mutant mice, in which spermatogenesis is arrested at different stages (Fig. 1F; Lyon & Searle 1989, Chubb 1992, Zimmermann *et al.* 1999). Further immunoblot analysis was performed with protein extracts isolating from embryonic stem cells, spermatogonial stem cells (Guan *et al.* 2006), Sertoli cell line 15P-1 (Rassoulzadegan *et al.* 1993), HeLa, and hepatoma Hep2G cell line. HSPA4 was expressed in embryonic, spermatogonial stem, and

Sertoli cell lines, whereas its expression levels were lower in both HeLa and Hep2G (Fig. 1G). These results suggest that HSPA4 is expressed in somatic and germ cells of testis. To determine the cellular localization of HSPA4, immunocytochemical analysis was performed on sections of fetal and newborn testis and ovary after embryonic day 13.5. We observed that HSPA4 is highly enriched in male gonocytes of E13.5, whereas lower levels of HSPA4-immunostaining were seen in somatic cells (Fig. 2A). Expression of HSPA4 remains at high levels in gonocytes throughout fetal stages (Fig. 2B and C). After migration of gonocytes from central to peripheral layer of seminiferous tubules during neonatal development and their start to differentiate to differentiated spermatogonia in 5-day-old testis, HSPA4 still expressed but at lower level than in fetal gonocytes (Fig. 2D). In adult testis, the expression of HSPA4 is slightly increased in post-meiotic germ cells (Fig. 2E). The HSPA4-specific immunostaining was confirmed by the absence of HSPA4-immunostaining in adult *Hspa4*-deficient testis (Fig. 2F). Immunofluorescence with antibodies directed against undifferentiated spermatogonia marker PLZF revealed that HSPA4 and PLZF are expressed in the same population of spermatogonia (Fig. 2G–I). When the preleptotene spermatocytes enter meiosis in 10-day-old testis, differentiated

Figure 2 Cellular localization of HSPA4 during testis and ovary development. (A–F) Immunohistochemistry of paraffin sections with anti-HSPA4 shows an abundance of HSPA4 in the gonocytes of wild-type testes at E13.5 (A), E15.5 (B), E18.5 (C), and in spermatogonia at P5 (D). In 3-month-old wild-type testis, expression of the HSPA4 is slightly increased in post-meiotic germ cells (E). A negative control of immunohistochemistry is the absence of HSPA4-specific immunostaining in adult *Hspa4*-deficient testis (F). (G–I) Expression of the HSPA4 in undifferentiated spermatogonia of wild-type mice is confirmed by immunofluorescence of testis at P5 with anti-HSPA4 (G) and anti-PLZF (H). HSPA4 and PLZF are expressed in the same population of spermatogonia (arrows). HSPA4 (red fluorescence) and PLZF (green fluorescence) are shown separately (G and H) and merged (I). DAPI (blue fluorescence) was used for nuclear staining (I). (J–L) Immunohistochemistry with HSPA4 antibody was performed on ovary sections of E13.5 (J), E15.5 (K), and E18.5 (L). Scale bar A–F and J–L = 50 μ m; G–I = 20 μ m.

spermatogonia, leptotene, and zygotene spermatocytes are expressed HSPA4 at very low levels (data not shown). In prenatal ovary, oogonia of E13.5 show weak staining (Fig. 2J), whereas HSPA4 is highly expressed in oogonia of E15.5 and E18.5 (Fig. 2K and L). The high enrichment of HSPA4 in gonocytes and oogonia throughout their embryonic development suggests that the absence of HSPA4 protein might directly affect the development of germ cells in both male and female embryos.

Hspa4 deficiency results in impaired male fertility

To investigate the function of HSPA4, we inactivated the *Hspa4* in mouse ESC. *Hspa4*-targeting vector was designed to replace a 3.0 kb genomic fragment containing exon 1 by neomycin resistance gene (Fig. 3A). Recombinant *Hspa4*^{+/-} ESCs were analyzed by Southern blot hybridization (Fig. 3B) and then used to generate chimeric mice. Chimeric mice were intercrossed with C57BL/6J females to establish the *Hspa4* mutant allele on a C57BL/6J×129/Sv hybrid genetic background. Interbreeding of heterozygous mice yielded a normal Mendelian ratio of *Hspa4*^{+/+}, *Hspa4*^{+/-}, and *Hspa4*^{-/-} offspring. These results indicate that there is no lethality caused by the *Hspa4* mutation. Male and female *Hspa4*-deficient mice developed into apparently normal adults. In *Hspa4*-null allele, exon 1 containing the translation initiation codon ATG is deleted.

Therefore, we expected that the targeted *Hspa4* allele would be transcribed into an untranslated *Hspa4* mRNA. The inactivation of *Hspa4* was confirmed by RT-PCR and northern and western blot analyses. The 596 bp cDNA probe recognized a weak band in RNA from *Hspa4*^{-/-} testis (Fig. 3C). RT-PCR with primers containing sequences of exons 1 and 3 was not able to amplify the *Hspa4* cDNA fragment from testicular RNAs of *Hspa4*-deficient mice (Fig. 3D). At protein level, the HSPA4 antibody recognized the expected 96 kDa HSPA4 protein in testes of wild-type and heterozygous animals, whereas the corresponding protein band was not detected in testes of *Hspa4*-deficient mice (Fig. 3E). These results confirm that the targeted disruption of *Hspa4* generated a null mutation.

To study the consequence of *Hspa4* mutation on female and male fertility, we intercrossed 13 males and seven females from F2 generation with wild-type mice of strain CD1 over a period of 3 months. All mating of *Hspa4*-deficient females were reproductive, and the average litter size (9.2 ± 2.4 , $n=18$) was not significantly different compared to breeding with wild-type females (9.6 ± 1.6 , $n=15$). Breeding of male mutants revealed that fertility was heterogeneous among males. Of the 13 males, eight did not produce a single litter, whereas the remaining five produce litter size (11.1 ± 3.4 , $n=16$) similar to those of their wild-type littermates (14.6 ± 1.2 , $n=15$).

Figure 3 Targeted disruption of the *Hspa4* gene. (A) Schematic representation of wild-type, targeted vector and recombinant allele. Exons are represented as numbered boxes. A 3.0 kb genomic fragment containing exon 1 was replaced by *pgk-neo* selection cassette (NEO). TK box represents thymidine kinase. The 3' external probe used and the predicted length of XbaI restriction fragments in Southern blot analysis are shown. The primers P1, P2, and P3 used to amplify the wild-type and mutant alleles by PCR are also indicated. Restriction sites relevant for generation of knockout vector and for screening strategies are SpeI (S), XhoI (X), XbaI (Xb), BamHI (B) and EcoRI (E). (B) Southern blot analysis of recombinant ES cell (ESC) clones. Genomic DNA from ESC clones was digested with XbaI and probed with the 3'-probe shown in panel A. (C) Northern blot with testicular RNA of *Hspa4*^{+/+}, *Hspa4*^{+/-}, and *Hspa4*^{-/-} mice was hybridized with the 596 bp *Hspa4* and β -actin cDNA probes. (D) RT-PCR analysis using testicular RNA and primers located in exons 1 and 3 of *Hspa4* confirmed the absence of exon 1 in *Hspa4* targeted transcripts. Amplification of *Hprt* cDNA in assays with RNA of three genotypes was used as a control. (E) Western blot with whole protein extracts from testes of *Hspa4*^{+/+}, *Hspa4*^{+/-}, and *Hspa4*^{-/-} mice was probed with HSPA4 and α -tubulin antibodies.

To verify whether the *Hspa4* deficiency results in disruption of spermatogenesis and/or sperm motility, we analyzed the number and the motility of spermatozoa collected from the cauda epididymides of 5-month-old wild-type, fertile, and infertile *Hspa4*^{-/-} males. A significant reduction in the mean number of spermatozoa was found in *Hspa4*^{-/-} males. Analysis of sperm motility and progressive movement showed significant differences only between spermatozoa of wild-type and infertile *Hspa4*^{-/-} mice (Table 1).

Spermatogenesis is arrested at meiotic prophase stage

Testis weights of 5-month-old infertile *Hspa4*-null mice (51.1 ± 12.1 mg, *n* = 5) were significantly reduced than those of control males (125.4 ± 4.2 mg, *n* = 5; Fig. 4A). To elucidate the cause of the reduction in number of spermatozoa, we analyzed cross sections of testes from 5-month-old wild-type and *Hspa4*-deficient mice. Testes of infertile mutant mice exhibited a diverse range of defects, varying in severity among males. Most seminiferous tubules were markedly smaller than those of wild-type controls (Fig. 4B and C). Tubules of testes from infertile mice contained Sertoli and early stages of spermatogenic cells; however, many pachytene spermatocytes have degenerated nuclei, and round and elongated spermatids were absent in most seminiferous tubules (Fig. 4D and E). Many tubules were vacuolated due to spermatocytes loss. Multinucleated spermatids, which may arise by widening of the intercellular bridges after meiotic division (Dym & Fawcett 1971), were frequently observed (Fig. 4B and C). Consequently, the mutant epididymides contained a few number of sperm and immature germ cells with compact chromatin were present (Fig. 4F and G).

To identify the spermatogenic stage, at which spermatogenesis is affected by *Hspa4* deficiency, testicular sections from different postnatal days were histologically and immunohistologically analyzed. No apparent differences were observed in the histological structure of seminiferous tubules between mutant and wild-type mice at postnatal days 5 and 10 (data not shown). Using the HSPH1 antibody to label gonocytes, the *Hspa4*^{-/-} and *Hspa4*^{+/+} tubules were observed to

contain equivalent numbers of gonocytes, suggesting that the *Hspa4* deficiency does not impair gonocytes or development of gonocytes to spermatogonia (Supplementary Figure 1, see section on supplementary data given at the end of this article). At postnatal day 10, the first spermatocytes are formed. The number of germ cells stained with anti-GCNA1, a marker of pre-meiotic and meiotic germ cells, in mutant and wild-type tubules, was not significantly different at postnatal day 10, suggesting that mitotic division in mutant testes is not affected (Supplementary Figure 1). However, a few of the seminiferous tubules of *Hspa4*^{-/-} testes contained meiotic germ cells (pachytene spermatocytes) at postnatal day 15 (Fig. 5A and B). By immunohistological staining with anti-HSPA4L, which is highly expressed in germ cells from pachytene spermatocytes (Held et al. 2006), a reduction was observed in the mean number of HSPA4L-immunopositive cells per tubule in *Hspa4*^{-/-} compared with wild-type testes (Supplementary Figure 1). At P20, spermatogenesis has reached the stage of round spermatids in majority of wild-type tubules. In contrast, mutant tubules were almost completely devoid of round spermatids and contained much fewer number of pachytene spermatocytes (Fig. 5C and D). At day 25, when tubules of wild-type littermates showed elongated spermatids, *Hspa4*^{-/-} testes showed a severe depletion of germ cells. In *Hspa4*-deficient testes, very few tubules contained round spermatids as the most advanced germ cells (Fig. 5E and F). To examine whether disrupted spermatogenesis is due to the impairment of chromosomal pairing, we analyzed the formation of the synaptonemal complex in the mutant germ cells. No abnormalities in the chromosome pairing were detected as judged by the proper accumulation of SCPY3 on the synapsed chromosomes during pachytene stage (Supplementary Figure 1). These results suggest that the *Hspa4* deficiency resulted in either developmental delay or partial arrest of the first wave of spermatogenesis.

To investigate whether the observed loss of germ cells is a result of enhanced apoptosis in *Hspa4*-null mice, TUNEL assay was performed on testis section of 10-, 15-, 20-, 25-, and 150-day-old mice. In 5-month-old testes, there were significantly more TUNEL-positive cells in seminiferous tubules of infertile *Hspa4*-null mice than in those of wild-type littermates (Fig. 6G and H). During postnatal development of testis, there were no significant differences in the number of TUNEL-positive cells between *Hspa4*^{+/+} and *Hspa4*^{-/-} testes at P10 (data not shown). A significant increase of TUNEL-positive spermatocytes was found in *Hspa4*-null mice at P15, P20, and P25 (Fig. 6A–F and I). These results indicate that germ cells at meiotic stages appear to be the most affected cells in *Hspa4*-deficient testes.

We then analyzed the expression of different meiotic and post-meiotic marker genes in testes of wild-type, fertile, and infertile mutant mice (Fig. 7A and B).

Table 1 Sperm analysis of *Hspa4*^{+/+} and *Hspa4*^{-/-} mice.

Genotype of mice	No. of sperm in cauda epididymis (10 ⁷)	Sperm motility (%)	Progressive motility (%)
<i>Hspa4</i> ^{+/+}	2.0 ± 0.1 (5)	63.2 ± 4.4 (5)	42.1 ± 5.3 (5)
<i>Hspa4</i> ^{-/-}			
Fertile	1.1 ± 0.4* (4)	57.0 ± 6.5 (4)	33.5 ± 9 (4)
Infertile	0.2 ± 0.3*(5)	18.3 ± 11.4*(4)	9.8 ± 5.9*(4)

Data for sperm analysis represent the mean ± S.D. for the numbers of individual measurements indicated in parentheses. *Value in *Hspa4*^{-/-} mice is significantly different from that in *Hspa4*^{+/+} mice (*P* < 0.01 by Student's *t*-test).

Figure 4 Testes and spermatogenesis of *Hspa4*^{+/+} and infertile *Hspa4*^{-/-} mice. (A) Infertile *Hspa4*^{-/-} mice showed reduced testis size compared with *Hspa4*^{+/+} mice. (B–E) Histological sections from testes of 5-month-old wild-type (+/+) and *Hspa4*^{-/-} mice (–/–) were stained with hematoxylin and eosin (H&E). A strongly reduced number of pachytene spermatocytes and round and elongated spermatids are found in most seminiferous tubules of mutant mice (C and E) compared with wild-type mice (B and D). Furthermore, degenerated germ cells and vacuoles (arrowhead) and presence of multinucleated giant cells (g) are frequently found in testes of infertile *Hspa4*^{-/-} mice. P, pachytene spermatocytes; rSp, round spermatids; eSp, elongated spermatids. (F and G) H&E-stained sections of epididymides of 5-month-old mice. *Hspa4*-deficient epididymides contain a few number of sperm, and immature germ cells with compact chromatin were present (arrow). Scale bar (B and C) = 100 µm; (D–G) = 50 µm.

Expression of *Sycp3* gene encoding synaptonemal complex protein-3 is restricted to leptotene and zygotene spermatocytes (Lammers *et al.* 1994). Northern blot analysis revealed that the expression levels of *Sycp3* in testes of fertile and infertile *Hspa4*^{-/-} mice are similar to those in wild-type testes. In contrast, expression of testis-specific genes encoding the phosphoglycerate kinase-2 (*Pgk2*) and acrosin (*Acr*), which were reported to be expressed in pachytene spermatocytes (Goto *et al.* 1990, Kashiwabara *et al.* 1990, Kremling *et al.* 1991), was markedly reduced in testes of infertile *Hspa4*-null mice. Similar results were also obtained for transcript

levels of post-meiotic genes *Hsc70t* (Hsp70 homolog gene) and transition nuclear protein 2 (*Tnp2*; Kleene & Flynn 1987, Tsunekawa *et al.* 1999). These results confirm that the disruption of spermatogenesis in *Hspa4*-deficient mice occurred late in meiotic prophase I.

The mild phenotype in spermatogenesis of *Hspa4*-null mice may be due to overexpression of other members of HSP110 family. Therefore, we analyzed the expression of HSPA4L and HSPH1 in testes of fertile *Hspa4*^{-/-} mice. Western blot analysis did not reveal a marked increase in the expression of HSPA4L and HSPH1 in testes of *Hspa4*-null mice (Fig. 7C).

Figure 5 Delayed and disrupted first wave of spermatogenesis in *Hspa4* mutant mice. (A–F) Testicular sections from wild-type (+/+) and *Hspa4*-null mice (-/-) at postnatal days 15 (P15), 20 (P20), and 25 (P25) were stained with H&E. Arrowheads and arrows indicate pachytene spermatocytes and spermatids, respectively. Scale bar = 50 μm.

Discussion

This research describes the expression and physiological function of HSPA4 in germ cell development. Expression of HSPA4 is ubiquitously expressed in both somatic and germ cells of testis. However, the expression is highly enriched in male and female germ cells of prenatal gonads. Expression of HSPA4 in male gonocytes is gradually decreased after migration to the basal layers of seminiferous tubules and differentiation to spermatogonia. This preferential expression leads us to study the specific role of HSPA4 in germ cell development. Analyses of *Hspa4*-deficient mice revealed that all *Hspa4*-null mice on the hybrid C57BL/6J×129/Sv genetic background were born at Mendelian ratio and were apparently normal. Although expression of HSPA4 can be detected in all tissues of wild-type mice, male infertility was the most apparent phenotype for *Hspa4*-deficient mice of the second generation. Male infertility is histologically characterized by a decreased number of the post-meiotic germ cells and an increased number of germ cells undergoing apoptosis. *Hspa4* mutants display a disruption of the first wave of spermatogenesis in

juvenile testes by postnatal day 15, when the most advanced germ cells in the testes remain at the late pachytene spermatocyte stage. The histological results were confirmed by immunohistological and RNA analyses. These results showed the presence of an equivalent number of gonocytes in neonatal *Hspa4*-null testes and a lower percentage of mature spermatids. Expression of early meiosis-specific genes was not affected in *Hspa4*-deficient testes. In contrast, expression of marker genes for later stages of meiotic prophase I and for post-meiotic germ cells was downregulated in the absence of HSPA4. These results indicate that the *Hspa4* deficiency impairs the development of most germ cells in late prophase I.

Numerous proteins that are required for the development of male germ cells through meiotic and post-meiotic stages are mostly translated in the pachytene spermatocyte stage (Eddy & O'Brien 1998). Failure of molecular chaperones to direct correct folding of newly synthesized proteins might lead to accumulation of misfolded and damaged proteins in pachytene spermatocytes, which could prompt spermatocytes to undergo

Figure 6 Enhanced apoptosis during germ cell development of *Hspa4* null mice. (A–H) Histological sections of *Hspa4*^{+/+} (+/+) and *Hspa4*^{-/-} mice (-/-) at postnatal days 15 (P15), 20 (P20), 25 (P25), and 150 (P150) were subjected to TUNEL staining. TUNEL-positive cells are mainly pachytene spermatocytes as indicated by their nuclear size and their position in the seminiferous tubules. Scale bar (A–F) = 50 µm; (G and H) = 100 µm. (I) For quantification of TUNEL-positive cells/seminiferous tubule, sections of testes derived from two mice per genotype per stage were microscopically analyzed. Number of the TUNEL-positive cells per tubule in each microscopic field was determined. An average of 10–20 randomly microscopic fields were scored for each genotype and developmental stage (*n* = 100–150 tubules). Then, the mean number of TUNEL-positive cells per tubule and standard deviation were calculated for the examined fields. Paired comparisons of the TUNEL-positive cells/tubule in testis among *Hspa4*^{+/+} and *Hspa4*^{-/-} mice in each stage were performed to determine the statistical significance by using Student's *t*-test. Bar graph represents mean ± S.D. **P* < 0.05.

apoptosis, rather carry on with meiotic division. Based on the high similarity of HSP110 family members, we expected that the molecular chaperones, which also include the NEF members of HSP110 family, would be abnormally or partially affected in *Hspa4*^{-/-} mice. The relatively leaky phenotype of *Hspa4*-deficient mice led us to suggest that other members of HSP110 family can partially compensate for the loss of HSPA4 function. HSPA4L and HSPH1 are possible candidates, because both proteins are widely expressed and localized in the cytoplasm like HSPA4. Therefore, the possibility of functional compensation between these proteins would be the cause that *Hspa4*^{-/-} mice are viable and display normal development except for disruption of spermatogenesis. This phenotype is also not completely penetrant, because some *Hspa4*-deficient germ cells were able to progress through spermatogenesis. To check this possibility, we intercrossed *Hspa4*-deficient mice with previously described *Hspa4l* mutant mice to produce mice lacking both genes. We found that *Hspa4*^{-/-} *Hspa4l*^{-/-} double knockout mice developed pulmonary hypoplasia that subsequently caused neonatal death during the first day of life (unpublished data). These results suggest a redundant function for HSPA4 and HSPA4L in lung maturation.

Expression of some HSP proteins is inducible by environmental stress, but expression of others can be either constitutive or developmentally regulated (Dix *et al.* 1997). HSPA4 and HSPH1 are ubiquitously expressed proteins and become relatively enriched in gonocytes after colonization of gonads by primordial germ cells (Fig. 2 and our unpublished data). The enrichment of both proteins in germ stem cells suggests their significant role for male and female germ stem cells. The results showed that the gonocytes are not affected in *Hspa4*-deficient mice, suggesting a redundant function of both proteins in germ cell development. To our knowledge, there is no report describing abnormal spermatogenesis in *Hsph1*-deficient mice. In one study, *Hsph1*-null mice were normally fertile (Nakamura *et al.* 2008). We are, therefore, interested in determining the impact of deleting both HSPA4 and HSPH1 on germ cell development by generation and characterization of *Hspa4*^{-/-} *Hsph1*^{-/-} double knockout mice.

Several reports, which used microarray analysis to identify preferentially expressed genes in different stem cells, revealed that the *Hspa4* is highly expressed in embryonic and different tissue-specific stem cells, and its expression is downregulated in their differentiated counterparts (Ramalho-Santos *et al.* 2002, Bhattacharya *et al.* 2004). *Hspa4* was one of the 216 enriched genes that were found to be expressed at high levels in embryonic, neural, and hematopoietic stem cells. Our results showing HSPA4 expression in germ stem cells further confirm the requirement of HSPA4 function for germ cell development. Although the physiological role of molecular chaperones for self-renewal of stem cells is

Figure 7 Expression profile of different germ cell markers and members of HSP110 family in *Hspa4*^{-/-} testes. (A) Northern blot with total RNA from testes of *Hspa4*^{+/+} (+/+), fertile (-/-F), and infertile *Hspa4*^{-/-} (-/-IF) mice was sequentially hybridized with cDNA probes for the indicated genes. (B) Densitometry analysis for the expression levels of different spermatogenic markers showing in Fig. 7A was determined. Expression level was expressed as relative percentage, with 1.0 given as the expression of spermatogenic marker in wild-type testis. Expression level of *EF2* gene was used for normalization. (C) Expression patterns of other members of HSP110 family in testes of wild-type and *Hspa4*-null mice. Immunoblots were probed with the antibodies shown at the right margin.

not known, it is believed that molecular chaperones may protect stem cells from aging due to oxidative stress (Ramalho-Santos *et al.* 2002). *Caenorhabditis elegans* that have an extended life span have elevated levels of molecular chaperones and enzymes that process oxidative free radicals and appear to be resistant to environmental stresses (Finkel & Holbrook 2000).

The partial penetrance of male infertility among *Hspa4*-null mice on the hybrid C57BL/6J×129/Sv genetic background may be reflected by the segregation of genetic modifiers on the hybrid genetic background. Background-related differences in male infertility phenotype have been reported in other mutations (Pearse *et al.* 1997, Yu *et al.* 2000, Adham *et al.* 2001). We have observed increased incidence of male infertility among *Hspa4*-null mice in F2 generation, which contain a high level of inter-individual genetic variability. The

decreased incidence of male infertility observed in the subsequent generation suggests that *Hspa4*-null males in different genetic background differ in fertility and would impose a selection bias against that genotype of infertile male. The decreased incidence of spermatogenic phenotypes in the following generations has also been described in different knockout mouse lines (Anderson *et al.* 2008, Burnicka-Turek *et al.* 2009).

The generation of *Hspa4*-, *Hspa4l*- and *Hsph1*-deficient mice constitutes an initial step in the understanding of the physiological role of HSP110 family members in mammals.

Materials and Methods

Generation of HSPA4-null mice

The PAC clone (RPCIP711P18115Q2) containing the *Hspa4* locus was isolated from the 129/Sv genomic library (RZPD, Berlin, Germany). The targeting vector was designed by replacement of exon 1 containing the start codon ATG with the *Pgk-neo* cassette. The 6 kb *SpeI/XhoI* and 4.5 kb *BamHI/EcoRI* genomic fragments containing the sequences of 5'-flanking region and intron 1 of *Hspa4*, respectively, were isolated from the PAC clone and inserted on either side of *Pgk-neo* cassette of pPNT vector (Fig. 3A). The targeting vector was linearized with *NotI* and used for transfection of RI ES cells. Recombinant ES-cells were checked for homologous recombination by Southern blot analysis. Genomic DNA was isolated from ES cells, digested with *XbaI*, separated in 0.8% (w/v) agarose gels, and transferred onto nylon membrane (Amersham Pharmacia). A 0.7 kb fragment located at 3' of targeting vector was amplified, radioactive labeled, and used as probe for the Southern blot analysis. Correctly targeted ES cell clones were injected into blastocysts derived from C57BL/6J mice and transferred into pseudo pregnant DBA/Bl6 females to generate chimeric mice. The chimeric founders were mated with C57BL/6J to generate heterozygous *Hspa4*^{+/-} mice, which were intercrossed to produce homozygous *Hspa4*^{-/-} mice. Genotyping of mice was carried out by PCR amplification of tail DNA. A 535 bp PCR product from the wild-type allele was detected using primer F1: 5'-GATCACGGGAAGT-GAGTGGT-3' and R1: 5'-GAGCGGGAG TGAGACAGTTC-3'. The targeted allele yielded a 274 bp product with primer F1: and primer PGK 5'-GGATGTGGAATGTGTGCGAGG-3'. The thermal cycling was carried out for 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Göttingen.

Northern blot analysis and RT-PCR

Total RNA was extracted using RNeasy mini-kit (Qiagen) and resolved (10 µg/lane) on an agarose gel containing 2.2 M formaldehyde and transferred onto nylon membrane. Blots were hybridized with 358 and 596 bp cDNA fragments containing the sequences of the C-terminal coding and the 3'-untranslated regions of *Hspa4*, respectively. The followed

primers were used to amplify the 358 and the 596 bp cDNA probes: 5'-GAAGAACTAGGGAAGCAAATCC-3' and 5'-TCAATGTCCATCTCAGGAAGC-3'; 5'-GTCCTGTTAAGAGCCCAGCTA-3' and 5'-ATTACCAT GCCTACACCCAAC-3'.

RT-PCR assay was performed using 2 µg total RNA and a one step RT-PCR kit (Qiagen). Primers to amplify *Hspa4* and *Hprt* transcripts were 5'-GTCCGTGGTGGGCATAGAC-3' and 5'-TTTATGCCCCGTTAATCCAGTG; 5'-GTCAAGGGCATATCCAACAA-CAAAC-3' and 5'-CCTGCTGGATTACATTAAAGCACTG-3', respectively.

Densitometry analysis was performed using the ImageJ Software (NIH, Bethesda, MD, USA); optic density for expression levels of *EF2* in northern blot analysis was used for normalization.

Fertility test and spermatozoa quality

To examine the fertility of *Hspa4*-deficient males on a hybrid 129/Sv×C57BL/6J genetic background, mature *Hspa4*^{-/-} males from the second generation were intercrossed, each with two wild-type CD1 females, for at least 3 months. The number and size of litters sired by each male were determined in a 3-month mating period.

Epididymides of ten *Hspa4*^{-/-} and four wild-type males were collected and dissected in IVF medium (MediCult, Jyllinge, Denmark). Sperm number in cauda epididymides was determined using the Neubauer cell chamber. To determine the sperm motility, spermatozoa were incubated for 1.5 h at 37 °C, 5% CO₂. Sperm suspension (10 µl) was transferred to the incubation chamber, which was set to 37 °C. Sperm movement was quantified using the CEROS computer-assisted semen analysis system (version 10, Hamilton Thorne Research, Beverly, MA, USA).

Histological and immunohistochemical methods

For histological analysis, tissues were fixed in Bouin's solution and embedded in paraffin. Sections (6 µm) were stained with hematoxylin and eosin (H&E). For immunohistochemistry, sections were preincubated for 1 h with 5% normal goat serum in 0.05% (v/v) Triton-X-100-PBS; incubated overnight at 4 °C with either rabbit anti-HSPA4 (N-60; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200 dilution, anti-HSPA4 (N-96; Santa Cruz) at 1:200 dilution, mouse anti-germ cell nuclear protein (GCNA1) at 1:50 dilution, or anti-HSPH1 (Sigma) at 1:200 dilution; washed with PBS; and then incubated with alkaline phosphate-conjugated goat anti-rabbit antibody or anti-mouse (Sigma) at 1:500 dilution for 1 h at room temperature. After washing with PBS, immunoreactivity was detected by incubation of the sections in a solution containing Fast Red TR/naphthol AS-MX phosphate tablets (Sigma). For PLZF and HSPA4 double immunofluorescent staining, sections of 5-day-old testes were incubated overnight at 4 °C with rabbit anti-HSPA4 and mouse anti-PLZF (D-9, Santa Cruz) antibodies. Sections were washed and then incubated with Cy3-conjugated goat anti-rabbit and FITC-conjugated goat anti-mouse antibodies (Sigma) for 1 h at room temperature. After washing, sections were mounted with Vectashield

mounting reagent (Vector, Burlingame, CA, USA) prior to fluorescence microscopy (Olympus, Hamburg, Germany).

TUNEL-positive cells were detected using an ApopTaq peroxidase *in situ* apoptosis kit (Obiogene, Heidelberg, Germany) according to the manufacturer's instruction.

Western blot analysis

Tissues were sonicated in RIPA buffer (Santa Cruz). Protein lysates were cleared by centrifugation at 16 000 g at 4 °C for 20 min, and protein concentration was measured by the Bradford assay (Bio-Rad). Total cell lysate (20 µg) was then resolved in 15% (w/v) SDS-PAGE gel and electroblotted onto nitrocellulose membrane. After blocking with 5% (w/v) skimmed milk in PBS, blots were incubated with either the primary antibodies rabbit anti-HSPA4 (1:500, Santa Cruz), rabbit anti-HSPA4L (1:500, Santa Cruz), rabbit anti-HSPH1 (1:1000, Sigma), or monoclonal anti-α-tubulin (1:10000, Sigma) with skimmed milk in PBS overnight at 4 °C. After a washing step, blots were incubated with HRP-conjugated anti-rabbit or anti-mouse IgG (1:2000, Sigma). The detection of immunoreactivity was performed using enhanced chemiluminescence (Pierce Chemical, Rockford, IL, USA).

Statistical analysis

Paired comparisons of the different sperm parameters and the number of apoptotic cells/tubule in testis among *Hspa4*^{-/-} and *Hspa4*^{+/+} mice were performed for statistical significance by calculating means ± s.d. and Student's *t*-test.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0023>.

Declaration of interest

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

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