Temperature sensitivity of DNA double-strand break repair underpins heat-induced meiotic failure in mouse spermatogenesis

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Mammalian spermatogenesis is a heat-vulnerable process that occurs at low temperatures, and elevated testicular temperatures cause male infertility. However, the current reliance on in vivo assays limits their potential to detail temperature dependence and destructive processes. Using ex vivo cultures of mouse testis explants at different controlled temperatures, we found that spermatogenesis failed at multiple steps, showing sharp temperature dependencies. At 38 °C (body core temperature), meiotic prophase I is damaged, showing increased DNA double-strand breaks (DSBs) and compromised DSB repair. Such damaged spermatocytes cause asynapsis between homologous chromosomes and are eliminated by apoptosis at the meiotic checkpoint. At 37 °C, some spermatocytes survive to the late pachytene stage, retaining high levels of unrepaired DSBs but do not complete meiosis with compromised crossover formation. These findings provide insight into the mechanisms and significance of heat vulnerability in mammalian spermatogenesis.
Mammalian spermatogenesis is a heat-sensitive process that occurs under low-temperature conditions. In many mammalian species, the testes descend into the scrotum at a later stage after developing deep in the abdominal cavity. Compared with the body core, the scrotal temperature is maintained to 2–6 °C lower by means of heat radiation from the skin surface and counter-current heat exchange between arterial and venal blood flows. When testes experience temperature elevation, spermatogenesis is compromised, leading to infertility.

From a clinical perspective, heat vulnerability during spermatogenesis is a well-recognized problem related to a broad spectrum of risk factors underlying male infertility in humans. Cryptorchidism, a pathological condition in which the testes do not descend completely but remain in the abdominal cavity or inguinal canal, causes spermatogenesis failure. Varicocele, in which the heat exchange between the testicular artery and vein is affected, is also a significant cause of male infertility. Testicular temperatures are increased in these conditions, whose surgical correction can improve spermatogenesis and fertility. In these high-temperature situations, the stage and extent of spermatogenesis defects are variable, and the precise temperature dependence and mechanisms underlying such defects remain largely unresolved.

Various in vivo experiments have been used to study heat sensitivity in mammalian spermatogenesis. In a classic study using pigs, naturally occurring cryptorchid testes recovered spermatogenesis when cooled by circulating water, demonstrating that high temperatures cause spermatogenesis defects. Artificial cryptorchidism, that is, surgical translocation of the testis into the inguinal canal, causes spermatogenesis failure. Varicocele, in which the heat exchange between the testicular artery and vein is affected, is also a significant cause of male infertility. Testicular temperatures are increased in these conditions, whose surgical correction can improve spermatogenesis and fertility. In these high-temperature situations, the stage and extent of spermatogenesis defects are variable, and the precise temperature dependence and mechanisms underlying such defects remain largely unresolved.

Artificial cryptorchid testes exhibit variable defects. In severest cases, all germ cells except undifferentiated spermatogonia are lost within a couple of weeks after surgical translocation, indicating that the transition from undifferentiated-to-differentiating spermatogonia is blocked. Such severe outcomes occur reproducibly if surgery follows a rigorous method, with the testes wrapped by the associated fat pad and positioned deep in the abdomen. However, less thorough surgeries result in inconsistent testicular position and variable defects wherein, in some cases, only spermatogonia and spermatocytes remain, while round spermatids may persist in others. A similar variability occurs in human testes with varicocele. Thus, heat may affect multiple steps of spermatogenesis, possibly at different temperatures. However, the limitations of artificial cryptorchidism hinder the detailed understanding of temperature sensitivity. Foremost, the testicular temperature cannot be reliably measured or controlled. Furthermore, the effect of extratesticular factors mediated by other organs, hormones, or the nervous system cannot be excluded.

Transient heat shock, which provides consistent results in short time courses, has been used to elucidate the mechanisms of spermatogenesis heat impairment. Lower-body bathing of mice in hot water (e.g., at 43 °C for 15 min) elicits stress response pathways and acute loss of spermatocytes and spermatids. However, such experiments cannot measure or control the actual testicular temperature or exclude extratesticular factors, either. Furthermore, it is unclear if the results of transient heating at non-physiologically high temperatures can be extrapolated to chronic exposure to temperatures around the body core.

Previously, we developed an ex vivo air-liquid interface culture of mouse testicular explants. This setting supports the entire process of spermatogenesis over several weeks, leading to the production of functional sperm, albeit less efficiently than that produced in vivo. Using this culture, we investigated the chronic effect of precisely controlled temperatures on mouse spermatogenesis under conditions free from extratesticular factors.

Results

High temperature impairs mouse spermatogenesis ex vivo. First, we measured the scrotal and body core temperatures of adult mice under normal conditions using minimally invasive thermometers. The measured average temperatures were 34.0 °C and 37.8 °C in the scrotum and body core, respectively, establishing the reported low-temperature condition in the scrotum (Fig. 1a and Supplementary Fig. 1a).

Based on these measurements, we evaluated the temperature dependence of spermatogenesis in ex vivo cultures. Testis explants prepared from postnatal day 4 (P4) mice were cultured in an air-liquid interface (Fig. 1b) at controlled temperatures with an accuracy of ±0.2 °C. At 34 °C, the culture supports spermatogenesis following essentially the same time course as in vivo development. To monitor the progression of spermatogenesis in the same explant during culture, we used Acr-GFP mice as the donors. Green fluorescent protein (GFP) expression was activated in these mice during meiotic prophase I under an acrosin promoter. Precisely, GFP expression starts in pachytene spermatocytes that are in stage IV seminiferous epithelium and continue through subsequent steps of meiosis and the formation of round and elongating spermatids (Fig. 1c, d).

The culture schedule used in this study is summarized in Fig. 1b. Initially, P4 testes contained only thin seminiferous tubules harboring immature spermatogonia (Fig. 1b, inset). During the 5 weeks of culture, the media was changed once every week, and seminiferous tubules grew and enlarged over the periphery of the explants. As described previously, the Acr-GFP signal appeared in these seminiferous tubules, indicating that spermatogenesis had progressed to the late pachytene spermatocytes (stage IV or later) (Fig. 1d), and the central region became necrotic due to a limited supply of oxygen and nutrients (Fig. 1c, Supplementary Fig. 1d). Based on the Acr-GFP signal in the peripheral region observed by fluorescence microscopy, we scored spermatogenesis progression qualitatively from grade 0 to 4 once every week. In grade 0 explants (GFP signal undetected), spermatogenesis did not reach the late pachytene stage. Grades 1–4 reflect the extent of spermatogenesis progression beyond this stage (Supplementary Fig. 1b). After 5 weeks, the explants were fixed and processed for hematoxylin-eosin (HE) staining and immunofluorescence (IF) to examine marker protein expression, including MVH (pan-germ cells), SCP3 (spermatocytes), KIT (differentiating spermatogonia), GFRα1 (a fraction of undifferentiated spermatogonia responsible for stem cell activity), and GATA4 (Sertoli cells) (Fig. 1c, d).

At 34 °C (the scrotal temperature), the explants showed Acr-GFP fluorescence at nearly full grade from 2 to 5 weeks, as reported previously. In most cases, all stages of spermatogenic cells (i.e., spermatogonia, spermatocytes, and round and elongating spermatids) developed in the peripherally located tubules, albeit less densely than in vivo-grown testes. These findings are in line with the IF results, demonstrating the presence of many MVH+ germ cells, SCP3+spermatocytes, KIT+ and GFRα1+spermatogonia, and GATA4+Sertoli cells. In contrast, at 38 °C (body core temperature), no Acr-GFP fluorescence was detected throughout the culture period. Consistently, we observed spermatocytes, but no round or elongating spermatids, in sections prepared at the end of the culture. The number of SCP3+spermatocytes and MVH+ germ cells was lower than in the explants cultured at 34 °C (Fig. 1d, Supplementary Fig. 1d-i). In contrast, GFRα1+ and KIT+spermatogonia and GATA4+Sertoli cells were abundantly observed at 38 °C, as well as 34 °C (Fig. 1g). These findings indicate that spermatogenesis was
Fig. 1 Ex vivo testis explant culture and heat sensitivity of mouse spermatogenesis. a Scrotal and abdominal temperatures in mice measured using an implanted thermometer, shown as a schematic diagram in the left panel. Mean ± SD of body core and testis temperatures measured in 14 individuals and averaged over the times of a day are shown in the right panel (see Supplementary Fig. 1a). *p < 0.01. b Outline of the ex vivo culture experiments and a representative image of a P4 Acr-GFP mouse testis. Scale bar, 20 μm; SG spermatogonia, IF immunofluorescence, HE hematoxylin-eosin staining. c Representative fluorescence microscopic images of a testis explant harboring Acr-GFP transgene after five weeks of culture as shown in b. Bright field (left) and fluorescence (right) images are shown. Scale bars: 500 and 250 μm in upper and lower panels, respectively. GFP⁺ seminiferous tubules were observed in the periphery while the central part was necrotic. d Schematic representation of mouse spermatogenesis and the expression of marker proteins used in this study. Early spermatocytes (Acr-GFP⁻) include leptotene, zygotene, and pachytene before stage IV, while the late spermatocytes (Acr-GFP⁺) include pachytene after stage IV and diplotene. e Acr-GFP fluorescence images of testis explants after five weeks of culture at 34 °C and 38 °C (left), and the summary of their GFP grade (0–4) over five weeks of culture at 34 °C (N = 99) and 38 °C (N = 51) (right). See also Supplementary Fig. 1c–d. Scale bar, 500 μm. f, g Representative images of sections of Acr-GFP mouse (5 week old) testes (in vivo) and the testis explants cultured for five weeks at 34 °C and 38 °C, stained with hematoxylin-eosin (f) and for indicated marker proteins (g). In (f), enlarged images are shown on the right. In (g), white and yellow arrowheads indicate GFRα1⁺ and KIT⁺ spermatogonia, respectively. SG spermatogonia, SC spermatocytes, RS round spermatids, ES elongating spermatids. Scale bars: 100 and 40 μm in (f, g), respectively.
impaired during the early spermatocyte stages in ex vivo culture at 38 °C.

Temperature-dependent spermatogenesis failure at multiple steps. To determine the temperature dependence of spermatogenesis, we incubated the explants at various temperatures ranging from 30 to 40 °C (Fig. 2a–c and Supplementary Fig. 2a). The variability in the progression of spermatogenesis between explants and within an explant prevented us from quantifying germ cells at different steps. Nevertheless, we observed reproducible and characteristic defects at specific temperatures (e.g., consistent absence of particular cell types) that are summarized in Fig. 2d and Supplementary Table 1.

We found that, among the temperatures tested, 34 °C provided the best conditions for spermatogenesis progression (Fig. 2a–d and Supplementary Fig. 2a). We observed all stages of germ cell development at 32 °C and 35 °C, although the number of elongating spermatids at these temperatures was fewer. At 36 °C, despite comparable Acr-GFP signals as at 34 °C, elongating spermatids were absent, and a few round spermatids were the most advanced germ cells (Supplementary Fig. 2b). At 37 °C, the Acr-GFP signal became weaker, no round or elongating spermatids were observed, and the Acr-GFP+ spermatocytes were the most advanced type of germ cells (Fig. 2a–d and Supplementary Fig. 1d–f and 2a). At 38 °C, as described earlier, no Acr-GFP signal was detected, while Acr-GFP− spermatocytes and spermatogonia remained. At 39 °C, the total germ cell number was reduced but the cellular composition was similar to that at 38 °C. At 40 °C, we observed no germ cells. Massive germ cell loss occurred at 30 °C. At all temperatures, GATA4+ Sertoli cells densely covered the inner surface of the tubules in the periphery of the explants, emphasizing that temperature sensitivity is characteristic of germ cells.

Therefore, using ex vivo culture, we found that spermatogenesis shows a delicate temperature dependence; it does not proceed beyond the stages of early spermatocytes, late spermatocytes, and round spermatids at 38, 37, and 36 °C, respectively.

High temperature compromises meiotic prophase I and causes apoptosis. We then explored the process of meiotic failure at 38 °C and 37 °C. In meiotic prophase I, spermatocytes establish synopsis of homologous chromosomes and crossover, through leptotene, zygotene, pachytene, and diplotene stages31. To determine which stage is impaired due to high temperatures and how, we prepared chromosome spreads from explants cultured at 34, 37, and 38 °C and in vivo-grown testes to stain for SCP3 and SCP1. SCP3 is an axial element protein that is localized to unpaired chromosomes and the synaptonemal complex of paired chromosomes. SCP1 is a transverse filament protein in the central element of the synaptonemal complex, localized on only the synapsed regions of the homologs32,33.

In leptotene, the axial element develops before synopsis, as detected by thin and often discontinuous SCP3 signals without overlapping SCP1. In zygotene, when homologous chromosome pairing and synopsis are established, SCP3 and SCP1 are distributed along the entire chromosome length and synapsed regions, respectively, exhibiting a characteristic Y-shape. In pachytene, when all homologues have fully synapsed and crossovers occur, SCP1 and SCP3 colocalize on the synaptonemal complex over the entire length of paired chromosomes. In diplotene, when the synaptonemal complex disassembles, partially synapsed homologue pairs with characteristic telomeric thickening of SCP3 staining were observed. Such localization of SCP3 and SCP1 was consistently observed in spermatocytes developed ex vivo at all tested temperatures (Fig. 3a). Further, co-staining with HORMAD1, an axial element protein associated with the unsynapsed region but not with the synapsed region, verified the validity of SCP3/SCP1 staining in these samples34 (Supplementary Fig. 3a–b).

Using these classification criteria, we examined the compositions of leptotene, zygotene, pachytene, and diplotene spermatocytes in ex vivo-grown testis explants (Fig. 3a, b). As expected, the explant cultured at 34 °C contained all of these stages, with proportions similar to those in vivo control. However, at 37 °C, only a small fraction of spermatocytes reached the pachytene and diplotene stages (Fig. 3b). At 38 °C, pachytene and diplotene spermatocytes were essentially absent, although leptotene and zygotene spermatocytes were preserved (Fig. 3a, b). These findings are in line with the undetectable and weak Acr-GFP signals at 38 and 37 °C, respectively (Fig. 2a).

We then investigated if and how spermatocytes are lost when culture temperature was raised (Fig. 3c and Supplementary Fig. 3c–d). After 2 weeks of culture at 34 °C, the explants showed a prominent Acr-GFP signal, indicating that spermatogenesis proceeded to late pachytene. Consistently, these explants harbored many SCP3+ spermatocytes. However, over 4 days after the temperature shift to 38 °C, the Acr-GFP signal disappeared, and the number of SCP3+ spermatocytes reduced considerably (Fig. 3c). During this period, cleaved Caspase-3, a specific indicator of apoptosis35, was detected in the SCP3+ spermatocytes (Fig. 3c and Supplementary Fig. 3d).

In summary, at 38 °C, spermatocytes do not complete chromosome pairing and die through apoptosis. At 37 °C, while many spermatocytes die, some survive until the pachytene and diplotene stages, but do not finish meiotic divisions to develop into spermatids.

DNA double-strand breaks increase at 37 °C and 38 °C. We further investigated the mechanisms by which meiotic prophase I failed. In the normal process (Fig. 4a), numerous DNA double-strand breaks (DSBs) are generated by the SPO11-TOPO6BL complex in the leptotene stage31,36. Then, RPAs are loaded to the DSBs by binding to the single-stranded DNA generated by end resection, to which the recombination proteins RAD51 and DMC1 assemble to form recombination foci37–42. In zygotene, RAD51 and DMC1 mediate homology-dependent DNA repair to establish synopsis between the homologous chromosomes. Chromosome pairing occurs synchronously, and its termination defines pachytene initiation. In pachytene, a minority fraction of DSBs gives rise to crossovers through a specific pathway of homologue-dependent DSB repair involving MLH1, such that each chromosome pair typically contains one (occasionally two or more) crossover(s)43. Crossover forms chiasmata as homologues detach in diplotene.

To determine how these processes are affected at different temperatures, we first evaluated the levels of DSBs by staining for phosphorylated histone H2AX (γH2AX)44. In leptotene and zygotene, newly formed DSBs elicit γH2AX generation, a process mediated by ATM45. γH2AX then decreases as the DSBs are repaired. Following this first wave, the second wave of γH2AX generation also occurs at later stages in response to unsynapsed chromosomes, known as the meiotic silencing of unsynapsed chromatids (MSUC)46. In in vivo samples, as reported, intense γH2AX signals were detected in the nuclei of leptotene and zygotene spermatocytes, but the signals were greatly reduced in pachytene, except for the intense signal in the XY-body reflecting the MSUC44 (Fig. 4b). These observations are consistent with the results of γH2AX quantification (Fig. 4c).

In testes grown ex vivo at 34 °C, γH2AX staining was similar to that of the in vivo control (Fig. 4b). At 37 °C, γH2AX signals were
Fig. 2 Spermatogenesis progression in testis explants cultured at a range of temperatures. a Representative Acr-GFP fluorescence images of testis explants after 5 weeks of culture at the indicated temperatures (left) and the summarized time course of Acr-GFP grades (right). N = 6, 4, 15, 9, 41, 11, and 11 for 30, 32, 35, 36, 37, 39, and 40 °C, respectively. Scale bar, 500 μm. b Representative images of HE-stained sections of testis explants cultured at the indicated temperatures. Rectangles in the left panels indicate the area magnified on the right. Scale bars, 100 μm. SG spermatogonia, SC spermatocytes, RS round spermatids, ES elongating spermatids. c Representative images of testis explant sections cultured at the indicated temperatures and immunostained for the indicated proteins. White and orange arrowheads indicate GFRα1+ and KIT+ spermatogonia, respectively. Scale bars, 40 μm. Some panels in a–c are also shown in Fig. 1e, f, and g, respectively. d Summary of the qualitative evaluation of spermatogenic cell types observed in the testes explants cultured for five weeks at the respective temperatures. −, never observed; +, observed infrequently but reproducibly; ++, found easily in some peripheral tubules; ++++, observed in most peripheral tubules as a group of cells; ++++, always observed as a robust population close to in vivo samples; ++++, observed as a completely developed population at levels of in vivo-developed testis, which never occurred in our ex vivo cultures.
Fig. 3 Meiotic prophase I progression and apoptosis in spermatocytes in ex vivo culture at different temperatures. a Representative images of chromosomal spreads at different stages of meiotic prophase I, prepared from in vivo-developed testes of 5-week-old Acr-GFP mice and from testis explants cultured at 34, 37, or 38 °C for 5 weeks as indicated. Samples were stained for SCP3, SCP1, and DNA (Hoechst 33342). Scale bars, 10 μm. 

b Proportions of spermatocytes in the leptotene (lep), zygotene (zygo), pachytene (pachy), and diplotene (diplo) stages, found in explants cultured for 5 weeks at the indicated temperatures classified visually on the chromosome spreads after immunofluorescence-staining for SCP1 and SCP3 as (a), according to the criteria described in the text. Values obtained from chromosome spreads prepared from pooled testicular cells of two in vivo-developed testes from different individuals and those from 6 to 7 ex vivo-grown explants are summarized. The total number of spermatocyte nuclei counted is indicated at the upper right of each panel. Percentages of spermatocytes nuclei in which all the autosomes have completed synapsis (i.e., the sum of pachytene and diplotene spermatocytes) were 63, 52, 16, and 0.5% for in vivo and ex vivo samples at 34, 37, and 38 °C, respectively. 

c Detection of cleaved Caspase-3 (red) and SCP3 (green) in testis explants following the temperature shift from 34 to 38 °C. Double-stained images overlaid with DNA staining (gray) and the signals for cleaved Caspase-3 alone are shown in the upper and lower panels, respectively. Enlarged images at positions indicated by rectangles are also shown below. Yellow arrowheads, Caspase-3+/SCP3+ double-positive cells (the dying spermatocytes). Scale bars, 40 μm.
Fig. 4 Events in meiotic prophase I and evaluation of DNA double-strand breaks (DSBs) at different temperatures. a Schematic representation of the stages of progression and events during mouse meiotic prophase I, and the expression of key proteins assayed in this study. Early pachytene includes those before stage IV of seminiferous epithelium, while those at stage IV or later are designated as late pachytene. b Representative images of chromosome spreads of spermatocytes in meiotic prophase I substages prepared from the testes of 5-week-old Acr-GFP mice and testis explants cultured for five weeks at 34, 37, or 38 °C. Samples were stained for γH2AX (magenta) with SCP3 (cyan) used for staging. The γH2AX signals are also shown separately in the right panels. Scale bars, 10 μm. c Dot plots showing the levels of the γH2AX signal in each spermatocyte nucleus at the indicated substage quantified from immunofluorescence images as shown in (b). Each dot represents an individual spermatocyte with horizontal bars indicating the mean values. The number of spermatocytes analyzed are indicated above the plots (N.D. not detected). Cells prepared from four testes from different individuals, and 14, 15, and 15 explants cultured at 34, 37, and 38 °C, respectively, were pooled for each sample. Multiple comparisons were conducted using the Kruskal–Wallis test among all values within each category and gave p-values of 2.31E-34, 1.13E-19, 2.53E-19, and 3.76E-5 for leptotene, zygotene, pachytene, and diplotene, respectively, and using the Steel–Dwass test for combinations of two values within each category resulting in the p-values as indicated in the plots (*p < 0.05, **p < 0.01).
higher than at 34 °C in leptotene and zygotene. In the few surviving pachytene and diplotene spermatocytes, weak γH2AX signals remained on and outside the chromosomal axes (Fig. 4b). In agreement with this, the quantified levels of γH2AX were highly variable in leptotene and zygotene spermatocytes, ranging from being comparable to being greater than that at 34 °C and in vivo; and the pachytene and diplotene spermatocytes showed higher γH2AX levels than at that 34 °C and in vivo (Fig. 4c). In addition to the unrepaird DSBs, such abundant γH2AX levels in pachytene may also be due to that generated through the MSUC. At 38 °C, γH2AX increased further in leptotene and zygote, while no pachytene or diplotene spermatocytes were observed (Fig. 4b, c). To summarize, DSBs levels increase at 38 °C and, to a lesser extent, at 37 °C, implying enhanced generation or compromised repair.

**High temperature compromises the DSB repair machinery.** To determine whether DSB repair was impaired at high temperatures, we examined the localization of RPA2, RAD51, and DMC1. At 37 °C and 38 °C, the RPA binding to the single-stranded DNA, or the generation of recombination foci are compromised. At 37 °C, RAD51 and DMC1 foci numbers were slightly reduced at 37 °C, whereas comparable numbers of RAD51 foci in late leptotene and zygotene spermatocytes, a fraction of which harbored abnormally few foci (<50) (Fig. 4b, c). The others showed zero or intermediate numbers, reflecting those before or during the formation of MLH1 foci, respectively (Fig. 7d–g). In explants, we observed no MLH1 foci in aberrant pachytene at any of the temperatures tested (i.e., 34, 37, and 38 °C), even on the fully synapsed chromosomes (Fig. 7d). This observation suggests that aberrant pachytene spermatocytes do not activate the meiotic program that usually occurs in late pachytene after stage IV; this is in line with the absence of Acr-GFP expression at 38 °C (Fig. 1). In contrast, a fraction of fully synapsed pachytene spermatocytes surviving at 34 °C and 37 °C harbored MLH1 foci, indicating their progression to late pachytene. However, the number of MLH1 foci reduced to ~20–27 at 34 °C, and 17–25 at 37 °C (Fig. 7d–g). Given the 19 autosome pairs and a pair of sex chromosomes, chromosome pair(s) lacking MLH1 foci were often observed in pachytene spermatocytes grown at 37 °C (Fig. 7e). Thus, crossover formation was compromised in late pachytene spermatocytes developed ex vivo, subcritically at 34 °C and critically at 37 °C.

Involvement of LINE1 transposon is not supported. Finally, we examined the potential involvement of dysregulated transposon expression, considering the increase in DSBs in explants cultured at 37 °C and 38 °C (Fig. 4b, c). In Caenorhabditis elegans, a transient heat shock (at 34 °C for 2 h, compared with a standard culture temperature of 20 °C) elicits dysregulated expression of transposons and an increase in RAD51 foci in spermatocytes. Heat-induced transposon activation also occurs in other systems such as Arabidopsis. Furthermore, in mice, mutants deficient in the piRNA pathway exhibit dysregulation of transposons (typically LINE1) and apoptotic death of spermatocytes. In vivo-developed testes, we found LINE1-ORF1 protein expression in spermatocytes in a small fraction of seminiferous tubules (Supplementary Fig. 5). However, in explants cultured at 34 °C, LINE1 expression broadened for unknown reasons, but a temperature-dependent increase in LINE1 expression was not observed (Supplementary Fig. 5). To further investigate the relationship between temperature and LINE1 expression, we used an in vivo setting (i.e., artificial cryptorchidism) considering the low LINE1 expression in the normal condition of the scrotum (at ~34 °C). After surgical translocation of the testis to the abdominal cavity (at ~38 °C), germ cell death, including that of spermatocytes, occurs from day 3 onward. On day 2, therefore, the surviving spermatocytes are likely already impaired and about to die. However, we found that LINE1-ORF1 expression remained limited to a minor fraction of seminiferous tubules on day 2, contrary to the upcoming ubiquitous germ cell death.
These results indicate that high
temperature and LINE1 dysregulation are not parallel but
separable events, suggesting that LINE1 does not likely contribute
to heat-induced spermatogenesis failure.

Discussion
In this study, we investigated the temperature sensitivity of mouse
spermatogenesis using a testis explant culture that supports the
process of spermatogenesis. We found that spermatogenesis was
impaired at multiple steps in a temperature-dependent manner.
In particular, spermatogenesis did not proceed beyond early
spermatocyte, late spermatocyte, and round spermatid at 38, 37,
and 36 °C, respectively. Such delicate temperature dependence
may explain the varying defects observed in human testes with
varicocele7 and artificial cryptorchidism in mice9–13,64,65. Unex-
pectedly, however, the most severe defect of artificial cryptorch-
idism, which blocks the transition from undifferentiated to
differentiating spermatogonia12,13, did not occur ex vivo at any
temperature tested. The pathology of cryptorchidism may not be
a simple consequence of high temperatures and warrants further
investigation.

Further, we investigated meiosis failure observed at 38 °C and
37 °C and found an increase in DSBs and asynapsis. We reason
that the meiotic checkpoint, also known as the pachytene
synaptic checkpoint, determines the outcome of such damaged
cells. The meiotic checkpoint surveys primary spermatocytes with
abundant DSBs and asynapsis, prevents damaged cells from
progressing to late pachytene, and eliminates them through
apoptosis66–71. In mice, a variety of mutants defective in DSB
generation (e.g., Spo11–/–), single-stranded DNA binding pro-
teins (e.g., Rpa1 conditional knockout), DSB repair machinery
(e.g., Dmc1–/–), or their recruitment (e.g., Meilb2–/–, Brme1–/–,
or Brca2 hypomorph) result in apoptotic cell death at
pachytene72–75. Further, showing dysregulated transposon

Fig. 5 Evaluation of RPA foci formation at different temperatures. a Representative images of chromosomal spreads in meiotic prophase I substages,
prepared from the testes of 5-week-old Acr-GFP mice and testis explants cultured for 5 weeks at 34, 37, or 38 °C. Samples were stained for RPA2 shown in
green, with SCP3 (red) used for staging and DNA (blue). RPA2 signals are shown separately in the right panels. Scale bars, 10 μm. b Dot plots showing the
number of RPA2 foci associated with SCP3 + chromosome axes in each spermatocyte of the indicated substage prepared from the in vivo and ex vivo testis
samples. Each dot represents an individual spermatocyte with means indicated as horizontal bars. The number of spermatocytes analyzed are indicated
above the plots (N.D. not detected). Cells prepared from three testes from different individuals (in vivo) and 8 (34 °C), 8 (37 °C), and 8 (38 °C) explants
were pooled and analyzed. Multiple comparisons were conducted using the Kruskal–Wallis test among all values within each category and gave p-values of
0.88, 1.39E-3, 1.13E-11, and 0.02, respectively, for early leptotene, late leptotene, zygotene, and pachytene. The Steel–Dwass test was applied for
combinations of two values within each category resulting in the p-values as indicated in the plots (*p < 0.05, **p < 0.01).
Fig. 6 Evaluation of repair foci formation at different temperatures. **a, c** Representative images of chromosomal spreads in meiotic prophase I substages, prepared from the testes of 5-week-old *Acr-GFP* mice and testis explants cultured for 5 weeks at 34, 37, or 38 °C. Samples were stained for RAD51 (a), and DMC1 (c) shown in green, with SCP3 (red) used for staging and DNA (blue). RAD51 and DMC1 signals are shown separately in the right panels. Scale bars, 10 μm. **b, d** Dot plots showing the number of RAD51 (b), and DMC1 (d) foci associated with SCP3+ chromosome axes in each spermatocyte of the indicated stage prepared from the in vivo and ex vivo testis samples. Each dot represents an individual spermatocyte with means indicated as horizontal bars. The number of spermatocytes analyzed are indicated above the plots (N.D. not detected). Cells prepared from three, four, and two testes from different individuals (in vivo) and 14 and 4 (34 °C), 15 and 10 (37 °C), and 15 and 4 (38 °C) explants were pooled and analyzed in (b, d), respectively. Multiple comparisons were conducted using the Kruskal-Wallis test among all values within each category and gave *p*-values of 0.01, 4.75E-18, 1.68E-9, and 0.11 in (b) and 0.02, 9.08E-9, 2.20E-3, and 0.25 in (d), respectively, for early leptotene, late leptotene, zygotene, and pachytene. The Steel-Dwass test was applied for combinations of two values within each category resulting in the *p*-values as indicated in the plots (*p < 0.05, **p < 0.01).
expression associated with increased DSBs, various mutations in the piRNA pathway cause apoptosis at the meiotic checkpoint \(^{59–61}\).

Figure 8 summarizes the temperature-dependent meiosis failure and the role of meiotic checkpoints that we propose. At 34 °C, prophase I progresses in a largely normal manner, leading to the production of functional sperm capable of fertilizing eggs \(^{20}\), although there is a slight increase in DSBs and a small reduction in crossovers. At 38 °C, however, much greater DSBs occur at leptotene and zygotene, at least in part, due to defective DSB
The meiotic checkpoint is an evolutionarily conserved surveillance mechanism that eliminates damaged spermatocytes in various conditions. These include the meiosis block in inter-specific hybrids, which leads to hybrid sterility, a vital means of speciation. The current study suggests that the meiotic checkpoint eliminates heat-damaged spermatocytes. This observation echoes the fact that most mammals cool their testes in the scrotum or through other mechanisms. We propose that the meiotic checkpoint serves as a safety device, ensuring that only spermatozoa developed at low temperatures can fertilize eggs. Future investigations are warranted to understand the mechanisms of heat-induced meiosis failure.

Although defects in DSB repair are crucial for heat-induced meiotic failure, additional mechanisms may also be involved. In particular, the generation of DSBs can increase at high temperatures. In this regard, a considerable possibility is the dysregulation of transposons by heat, as recently demonstrated in C. elegans. In mice, while LINE1 is unlikely to contribute to the temperature-dependent increase in DSBs, the involvement of other transposons is an open question that warrants further investigation. The assembly of MLH1+ late recombination nodules or crossover formation might also be heat-sensitive. More generally, temperature effects may alter over time between the initial (acute) and late (chronic) phases owing to the hormesis effect. Thorough and careful investigations are necessary to fully understand the mechanisms of heat-induced meiosis failure.

By nurturing spermatogenesis at arbitrary temperatures, free from extratesticular factors, explant culture provides an invaluable and otherwise unavailable opportunity to study heat vulnerability in spermatogenesis. At the same time, the current culture supports spermatogenesis less efficiently than in vivo development, even at the optimum temperature (34 °C), which could modify the effect of temperature. Such inefficiency may reflect the subcritical defects observed at 34 °C, including expansion of LINE1 expression, a mild increase in DSBs, and a slight decrease in crossovers. These findings may help further optimize culture conditions and improve the usefulness of the explant culture.
were prepared and submerged in the culture medium Serum Replacement/1% Penicillin
at 34 °C, whereas some survived to late pachytene at 37 °C. All cells are eliminated at the meiotic
checkpoint at 38 °C, whereas some threshold survive to late pachytene. At 34 °C, meiosis is completed leading to spermatid formation. At
high temperatures (37 °C or 38 °C), DNA double-strand breaks (DSBs) increased, at least in part, due to defective DSBR repair, leading to increased
asynapsis. Heat may also increase the DSBR genesis. The degree of
impairment varies between cells, with severely affected cells falling into a state of aberrant pachytene and less-affected cells completing
chromosome pairing. The meiotic checkpoint then surveys the DSBs and asynapsis in these cells. Cells with DSBs and asynapsis above the threshold
(including aberrant pachytene and probably some fully synapsed early pachytene) are eliminated through apoptotic cell death; those below the
threshold survive to late pachytene. All cells are eliminated at the meiotic checkpoint at 38 °C, whereas some survived to late pachytene at 37 °C.
However, late pachytene spermatocytes surviving at 37 °C retain normally abundant DSBs and asynapsis, leading to compromised
crossover formation and cell death without completing meiotic divisions.

In conclusion, we hope that ex vivo culture and the findings of this study will help to better understand the mechanisms of temperature sensitivity in mammalian spermatogenesis. In addition to developing an infertility treatment strategy, the results would also provide insights into finding the answer to the fundamental question of why does mammalian spermatogenesis proceeds at low temperatures.

Methods

Mice. Acr-GFP transgenic mice, designated formally as B6;B6C3-Tg(Acr03-E GFP)01Osb (RIKEN BRC, #RBRC00886)21,22, with a genetic background of C57BL/6 J
were maintained under a 12 h light/12 h dark at room temperature (25 ± 2 °C).

Animal Care and Use Committee of the National Institutes of National Sciences.

Artificial cryptorchidism. Eight-week-old C57BL/6 J mice were subjected to operations of artificial cryptorchidism following a rigorous method wherein the testes were wrapped by the associated fat pad and positioned deep in the abdomen13.

Measurement of the scrotal and body core temperatures in mice. IPPT-300 transponders (BioMedic Data Systems) were implanted into the scrotal sac and peritoneal cavity (under the liver) of wild-type C57BL6/J mice. One week after transponders (BioMedic Data Systems) were implanted into the scrotal sac and peritoneal cavity (under the liver) of wild-type C57BL6/J mice. One week after
surgical, the temperature at each position was measured separately using a DIAS-7006S scanner (BioMedic Data Systems) every 2 h for 24 h. Ambient conditions were maintained under a 12 h light/12 h dark at room temperature (25 ± 2 °C).

Ex vivo culture of mouse testes explants under different temperatures. Ex vivo culture was performed as previously described20,21,22, with minor modifications.

Briefly, 1.5% (wt/vol in PBS) agarose gels (Dojindo Laboratory, Kumamoto, Japan) were prepared and submerged in the culture medium containing 10% KnockOut Serum Replacement/1% Penicillin-Streptomycin) for >24 h, repeatedly 2–3 times. Whole testes dissected from euthanized neonatal Acr-GFP mice (4 days postpartum) were removed from the tunica albuginea and, without cutting into multiple pieces, gently positioned on the agarose gel in a 12-well culture plate with 500 μl of the above-described medium. The medium was changed once a week for 5 weeks. The cultured incubators (APC-30DR; Astec) were supplied with 5% CO2 in the air and maintained at specific temperatures (ranging from 30 to 40 °C), controlled with an accuracy of ±0.2 °C.

The grading of spermatogenesis progression based on Acr-GFP expression was performed visually at the time of weekly medium change, using an IX73 inverted fluorescence microscope (Olympus). Grading was performed unblinded as the observation had to be completed in a short period to minimize the temperature fluctuation.

Histological analysis. Testes removed from euthanized mice or cultured testes explants were fixed overnight in Bouin’s solution or 4% PFA in PBS at 4 °C, embedded in paraffin, sectioned, and mounted on a glass slide. According to standard protocols, sections were stained with hematoxylin and eosin (H&E), or with hematoxylin and periodic acid-Schiff (H-PAS) and mounted for microscopy.

Immunofluorescence on sections. Immunofluorescence (IF) was performed on paraffin sections (7 μm thick) for most cases. For double staining of LINE1-ORF1 and SCP3 (Supplementary Fig. 5), samples were fixed overnight in 4% PFA in PBS at 4 °C, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek), cryosectioned (8–10 μm thick), and attached to MAS-GP type A coated slides (Matsunami).

Immunostaining was performed following the protocol described previously with minor modification89. Briefly, the sections were incubated with Blocking One Histo (Nacalai Tesque) solution for 1 h at room temperature, followed by incubation with primary antibodies (described below) in 4% donkey serum/Can Get Signal Solution 1 (Toyobo) overnight at 4 °C. After the sections were washed with 0.04% Tween 20 in PBS, they were incubated with appropriate Alexa Flour-conjugated secondary antibodies (Thermo Fisher Scientific and Jackson Immunoresearch) in 4% donkey serum/Can Get Signal Solution 2 containing Hoechst 33342 for 2 h at room temperature. After the slides were washed with the secondary antibody solution, they were mounted using Fluoro-KEEPER Antifade Reagent (Nacalai) for fluorescence microscopy.

The following primary antibodies were used at the indicated dilutions: anti-GFRα1 goat polyclonal antibody (R&D Systems, #AF560, 1:1000); anti-KIT goat monoclonal antibody (R&D Systems, #AF1356, 1:100), mouse anti-Vasa homolog (M35/1) rabbit polyclonal antibody (Abcam, #ab1340, 1:500); anti-SCP3 mouse monoclonal antibody (Abcam, #ab97672,1:1000), anti-GATA4 rabbit polyclonal antibody (Thermo, #PA1-102, 1:500); anti-cleaved Caspase-3 rabbit polyclonal antibody (Cell Signaling, #9661, 1:500); and anti-LINE1-ORF1 rabbit polyclonal antibody (a generous gift from O’Carroll D, The University of Edinburgh, Edinburgh, UK, 1:5000) (Supplementary Table 2).

Nuclear spread of spermatocytes. The nuclear spread of spermatocytes from in vivo-developed testes and cultured testes explants was prepared using the dry-down method as described previously21,22, with modifications. Briefly, the tissues were minced with scissors and then dissociated into single cells by vigorous pipetting and filtration through a nylon mesh with 70 μm pores (Becton Dickinson). Cells were then suspended in PBS for hypotonic treatment by mixing with an equal volume of 30 mM Tris, 50 mM sucrose, 17 mM trisodium citrate dihydrate, 5 mM EDTA, and 0.5 mM dithiothreitol. Cells were washed and resuspended in PBS, mixed twice with 100 mM sucrose solution (pH 8.2), and then with three volumes of 1% PFA/0.1% (v/v) Triton X-100 in 10 mM sodium borate, before being dried wet onto a glass slide. The slides were then incubated in a humidified chamber for 1 h at room temperature, and then air-dried and frozen at −80 °C before IF to obtain sufficient amounts of starting materials to avoid the massive loss because of their small size during these procedures, several (e.g., 4–8) ex vivo explants were pooled.

For IF, the specimens were first blocked with 10% or 2% fetal bovine serum in 0.05% Triton X-100 in PBS (blocking buffer) for 1 h at room temperature and then with primary antibodies (listed below) in blocking buffer overnight at 4 °C. The specimens were then washed with 0.05% Triton X-100 in PBS, and incubated with Alexa Flour-conjugated secondary antibodies diluted in blocking buffer containing Hoechst 33342 for 2 h at room temperature before they were washed and mounted for fluorescence microscopy.

The primary antibodies used are as follows: anti-DMC1 rabbit polyclonal antibody (Santa Cruz, sc-22767, 1:1000), anti-SYCP1 polyclonal antibody (Novus Biologicals, #NBU300-229, 1:500), anti-SCP3 rabbit polyclonal antibody (Abcam, #ab15093, 1:500); anti-SCP3 mouse monoclonal antibody (Abcam, #ab97672, 1:1000), anti-phospho-H2AX antibody (Ser139; Millipore, #07-164, 1:500), anti-
RPA2 (Cell Signaling Technologies, #2208, 1:80), anti-RAD51 (Bio Academia, #70-
002, 1:100), anti-MLH1 (BD Biosciences, #551092, 1:100), and anti-NORMAX1 (ProteinTech, #13917-1-AP, 1:1000) (Supplementary Table 2).

Image acquisition and processing. The sectional specimens on glass slides (HE, PAS-hematoxylin, or immunostained samples) were observed and photographed using a BX51 upright fluorescence microscope equipped with a DTF2 CCD camera operated using a cellsens imaging software (Olympus). Observations and signal quantification of γH2AX staining (Fig. 4b, c) were performed using a Leica SP8
confocal microscope. For explants, GFP fluorescence was observed and photographed using an IX73 inverted fluorescence microscope equipped with a DP72 CCD camera operated using cellSens imaging software (Olympus). Photoshop (Adobe) was used to adjust the signal intensity uniformly over each image, without losing the original information. An illustrator (Adobe) was used to create figure panels.

Statistics and reproducibility. Figure 1a: Both scrotal and abdominal temperatures were measured in 14 wild-type mice at 2 timepoints at 2 h intervals (16:00 to 14:00 on the next day). The scrotal and abdominal temperatures were averaged over the 12 timepoints for each mouse. Such averaged values (per individual) were further averaged among 14 mice, shown with SD. Statistical evaluation was performed using matched Student's t-test (p = 4.6E-11).

Figure 1e: The Acr-GFP grade (0–4) of testicular explants, determined visually under a fluorescence microscope once per week during the culture period of five weeks at 34 °C or 38 °C, is summarized in component bar charts. 99 and 51 explants were analyzed for 34 and 38 °C cultures, respectively.

Figure 2a: The Acr-GFP grade (0–4) of testicular explants, determined visually under a fluorescence microscope once per week during the culture period of five weeks at 34 °C or 38 °C, is summarized in component bar charts. The number of explants were 6, 4, 99, 15, 9, 41, 51, 11, and 11 for 30, 32, 34, 35, 36, 37, 38, 39, and 40 °C, respectively. Data for cultures at 34 °C and 36 °C are identical to those shown in Fig. 1e.

Figure 3b: Compositions of prophase I spermatocytes in different subcategories (leptotene, zygote, pachytene, or diplotene) were analyzed for 34 and 38 °C explants. The total number of spermatocytes counted was 420, 549, 496, and 456 in the in vivo-developed tests (2 tests from different individuals) and testis explants cultured for five weeks at 34 °C (6 explants), 37 °C (7 explants), and 38 °C (7 explants), respectively. The number of spermatocytes in the leptotene stage was 108, 139, 211, and 233, those in the zygote stage were 40, 105, 206, and 201, those in the pachytene stage were 200, 259, 56, and 2, and those in the diplotene stage were 63, 26, 23, and 0, for the in vivo-developed tests and testis explants cultured at 34, 37, and 38 °C, respectively.

Figure 4c: To quantify the H2AX signal, we acquired immunofluorescence images of meiotic chromosome spread prepared from in vivo and ex vivo testicular cells stained for SCP3 and SCP1 (using shown in Fig. 1a, 3b), using a BX51 upright confocal system (Leica). The H2AX signal intensity of individual spermatocytes was quantified as the sum of pixel values within a nucleus using the Fiji software49, with cells classified visually in the leptotene, zygote, pachytene, or diplotene stage based on the SCP3 staining pattern. Four tests from different individuals were pooled for each condition, whereas spermatocytes prepared from 14 (34 °C), 15 (37 °C), and 15 (38 °C) explants were pooled for each ex vivo sample to prepare the nuclear spread specimens. As indicated in the panel, the number of spermatocytes analyzed to be in the leptotene stage was 73, 59, 79, and 98, those in the zygote stage were 54, 62, 76, and 49, those in the pachytene stage were 89, 85, 71, and 0, and those in the diplotene stage were 68, 47, 45, and 0 for the in vivo and ex vivo explants at 34, 37, and 38 °C, respectively. In the dot plots, the values of the H2AX signal are shown in the AU. Multiple comparisons were conducted using the Kruskal–Wallis test among all values within each category, providing p-values of 2.31E-4, 1.13E-19, 2.53E-19, and 3.76E-5 for leptotene, zygote, pachytene, and diplotene stage, respectively. The Steel–Dwass test for combinations of two values within each category, resulting in p-values as indicated in (5b), 0.01, 4.75E-18, 1.68E-9, and 0.11 in (6b), and 0.88, 1.39E-3, 1.13E-11, and 0.02 in (5b), 0.01, 4.75E-18, 1.68E-9, and 0.11 in (6b), respectively. The number of spermatocytes was counted in the in vivo-developed tests and testis explants cultured at 34, 37, and 38 °C, respectively.

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5. Durante SC3P and SC3P1. Different spermatogenic stages are identified following authentic criteria, that is, the presence of SC3P1 chromosome axes and the absence of SC3P3/SC3P1+ synapsed regions. Normal zygote spermatocytes were newly classified as zygote spermatocytes (i.e., the presence of both SC3P3/SC3P1+ synapsed region and SC3P3/SC3P1+ synapsed region) but not as aberrant pachytene spermatocytes. Aberrant pachytene spermatocytes were in an unusual state of zygote (though confusingly, lack of one or more full synapsed chromosomes and one or more autosomes pairs that were unsynapsed or were partly synapsed in less than 50% of its length. Pachytene spermatocytes were defined as those that had fully synapsed except for the XY body. The total number of spermatocytes counted was 495, 334, 330, and 176 for in vivo-developed tests (prepared from four testes of different individuals) and testes explants cultured for five weeks at 34 °C (10 explants), 37 °C (15 explants), and 38 °C (10 explants), respectively. The resultant counts of leptotene, normal zygote, aberrant pachytene, and pachytene spermatocytes were 49, 36, 37, and 407 in in vivo-developed tests and 45, 47, 27, and 225 in in vivo-developed tests at 34 °C, 37 °C, 113, 142, 234, and 39 in explants at 37 °C, and 113, 36, 27, and 0 in explants at 38 °C, respectively.

7E The MLH1 foci associated with SCP3+ axes were counted manually in spermatocytes harboring at least one fully synapsed chromosome [including aberrant pachytene and (fully synapsed) pachytene spermatocytes], on chromosome spread stained for MLH1 and SCP3. Cells prepared from three testes of different individuals and those from 10 (34 °C), 12 (37 °C), and 12 (38 °C) explants were pooled and analyzed. The total number of spermatocytes counted were 158, 108, 158, and 50, respectively.

7F The number of MLH1 foci was counted in the same manner as described above for Fig. 7E. However, only pachytene spermatocytes harboring intense and discrete MLH1 foci were counted to assess the crossover formation in the surviving cells. Three testes of different individuals and those from 10 (34 °C), 12 (37 °C), and 12 (38 °C) explants were pooled to prepare the chromosome spread; 76, 39, 47, and 0 spermatocytes were analyzed in in vivo samples and explants at 34, 37, and 38 °C, respectively. The number of MLH1 foci per nucleus is summarized in dot plots, with the mean values shown by horizontal bars. Multiple comparisons were conducted using Kruskal–Wallis test, providing the p-values of 9.35E-13, and Steel–Dwass test for combinations of two values, resulting in the p-values as indicated in the panel.

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