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Postmeiotic modifications of spermatogenic cells are accompanied by inhibition of telomerase activity

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Abstract We investigated whether testicular telomerase activity is due to telomerase expression in all cells or expression in a limited number of cells. Telomerase activity was assayed in highly purified fractions of spermatogonia cells plus primary spermatocytes, secondary spermatocytes plus round spermatids, secondary spermatocytes plus spermatids plus spermatozoa, round spermatids, or spermatozoa prepared from healthy or cryptorchid animals. Telomerase activity was additionally assayed in testicular tissue of prepubertal animals and animals with Sertoli cell only pathophysiology. Telomerase activity was detected in fractions containing primary spermatocytes and/or secondary spermatocytes and/or spermatids. Fractions enriched in round spermatids were positive for telomerase activity. In contrast, spermatozoa or Sertoli cell fractions were negative for telomerase activity. Using the relative telomerase activity assay and the sensitive quantitative telomerase assay to quantify telomerase activity, we showed that induction of cryptorchidism does not result in quantitative alterations in testicular tissue telomerase activity. In addition, elimination of round spermatids does not lead to significant alterations in testicular tissue telomerase activity. The present results suggest that the male gamete telomerase activity is inhibited during spermiogenesis. Furthermore, it appears that spermatogonia/primary spermatocytes are the main sources of telomerase activity in the testis.

Key words Spermatocyte · Spermatid · Telomerase · Testis

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Introduction

Accurate chromosome transmission during cell division is essential for cell survival. When chromosome integrity is lost, cell death or unregulated growth may occur. Telomeres are repeated sequences located on both ends of individual chromosomes in eukaryotes [2, 3]. Telomeres stabilize natural chromosome ends and inhibit aberrant fusions and rearrangements that occur on broken chromosomes. Furthermore, they progressively shorten with age in somatic cells in vivo and in vitro. Thus, telomeres have attracted much attention as a mitotic clock signaling senescence [16, 7, 22]. In humans and all other vertebrates, telomeric DNA consists of tandem repeats of the highly conserved G-rich sequence TTAGGG [2]. Telomere repeats are synthesized de novo onto chromosome ends by the enzyme telomerase. Telomerase is a transcriptase containing an RNA template. The RNA template effectively allows telomerase to add telomeric sequences to the ends of newly replicated DNA. Telomerase activity is upregulated in a variety of immortal cell lines and tumors in both human and mouse [5, 17, 20]. Telomerase activity is expressed in most human tumor tissues, but not in most normal tissues, tissues adjacent to tumors, or benign growth [12]. Telomerase activity has also been demonstrated in fetal and adult human testes [28]. The telomerase hypothesis suggests that telomerase activity is high in embryonic cells and that it decreases in somatic tissues during development and differentiation [6].

The objective of the present study was to evaluate whether testicular telomerase activity is due to telomerase expression in all cells or expression in a limited number of cells. Furthermore, we attempted to investigate whether there are quantitative changes in telomerase activity of testicular tissue or in telomerase profiles of specific subpopulations of spermatogenic cells in animals with spermatogenic arrest at the primary spermatocyte stage or at the round spermatid stage. Such a study could provide information on whether the cascade

of biochemical events related to the development of arrest in spermatogenesis influences the mechanisms regulating telomerase activity.

Materials and methods

Telomerase activity was assayed in subpopulations of spermatogenic cells isolated from healthy B6D2F1 8-week-old mice ($n = 9$, group A1), 10-week-old mice ($n = 9$, group A2), 8-week-old cryptorchid mice (bilateral cryptorchidism was induced at the age of 4 weeks, $n = 12$, group B1), 10-week-old cryptorchid mice (bilateral cryptorchidism was induced at the age of 4 weeks, $n = 9$, group C1), and 10-week-old cryptorchid mice (bilateral cryptorchidism was induced at the age of 2 weeks, $n = 9$, group D) treated with adriamycin (20 mg/kg once per 10 days) and hydroxyurea (1.344 mg/kg once per 10 days) after the induction of cryptorchidism. In addition, telomerase activity was evaluated in testicular sperm samples and epididymal sperm samples prepared from healthy 10-week-old mice ($n = 5$, group A3).

Testicular tissue telomerase activity was also assayed qualitatively in eight healthy 10-week-old mice (group A4) and eight 10-week-old cryptorchid mice (group C2) treated in same fashion as those in group C1. In addition, the testicular tissue relative telomerase activity (RTA) was assessed in groups A4 and C2. As RTA was considered the number of cells required had to be sufficient to obtain a telomerase activity ladder of equivalent intensity to that obtained using a positive control sample (1000 human uterine cervical cancer cells) [9, 10]. Telomerase activity was also assayed qualitatively in a highly purified sample of round spermatids prepared from additional 8-week-old healthy mice (group A5, $n = 5$). Furthermore, testicular tissue RTA was estimated in 17-day-old mice (group A6, $n = 8$) and 22-day-old mice (group A7, $n = 8$).

Preliminary experiments have demonstrated that spermatogenesis is arrested at the primary spermatocyte stage in 17-day-old mice. In contrast, in 22-day-old mice spermatogenesis is arrested at the round spermatid stage. No elongating spermatids are observable in the latter mice. The two testicles from one animal were processed for mincing/telomerase assay in each of the above experiments. In additional experiments, a highly sensitive quantitative telomerase assay (SQTA) was applied to quantify the testicular tissue telomerase profiles of cryptorchid animals treated in the same fashion as those of group B1 (group B2, $n = 8$; 8 weeks old), or group C1 (group C3, $n = 8$; 10 weeks old). Furthermore SQTA was performed on the testicular tissue of healthy animals of the same age as groups A2 and A1 (groups A8 and A9, respectively, $n = 8$ in each group). Testicular tissue from the right testis only was processed for SQTA in groups B2, C3, A8 and A9. Finally further experiments were performed to compare quantitatively the telomerase activity of specific germ cell subpopulations within four groups of mice (A1-N, B1-N, C1-N, and A5-N; $n = 10$ in each group). Mice of groups A1-N, B1-N, C1-N, and A5-N were of the same age/underwent the same treatment as the mice of the groups A1, B1, C1, and A5, respectively. The two testicles from one mouse each from groups A1-N, B1-N, C1-N, and A5-N were minced and processed for separation of specific spermatogenic cell subpopulations. Subsequently SQTA was performed in each recovered cellular fraction.

Induction of cryptorchidism

Mice of groups B1, B2, B1-N, C1, C2, C1-N, and C3 were anesthesized by intraabdominal injection of Nembutal (35 mg/kg; Abbott laboratories, Tokyo, Japan). The abdominal cavity was opened longitudinally and both testicles were placed into the abdominal cavity and fixed to the abdominal wall muscles by placing a nylon suture into the tunica albuginea of each testis. Mice of groups A1, A2, A4, A8, and A9 underwent a sham-operation at the age of 4 weeks by placing both testicles in the abdominal cavity and passing a nylon suture through the tunica albuginea. The testicles of the sham-operated animals were replaced within the scrotum.

Preparation of fractions of spermatogenic cells

A small piece from the testicles of the groups A1, A2, A4, B1, B2, C1, C2, and D were processed for staining with hemotoxyline/eosin. Testicles from animals of groups A1, A1-N, A2, A5, A5-N, B1, B1-N, C1, C1-N, and D were minced in SOF medium [31]. Spermatogenic cells were dispersed out of the seminiferous tubules and filtered via an 11- to 13-μm pore size filter (Tomoda, Matsue, Japan). The sediment (S1) and a part of the filtrate (F1) were processed for qualitative telomerase assay (groups A1, A2, B1, C1, and D) or quantitative telomerase assay (groups A1-N, B1-N, and C1-N). Another part of the filtrate-F1 was filtered through an 8- to 10-μm filter.

Telomerase activity was assayed in the sediment (S2) and the filtrate (F2) qualitatively (groups A1, A2, B1, C1, and D) or quantitatively (groups A1-N and B1-N). F2 samples from groups A5 and A5-N were mixed with pronase (final concentration: 0.2 mg/ml; Calbiochem - Behring, La Jolla, Calif.) for 7 minutes. Pronase causes agglutination of mature spermatids and spermatozoa in sticky masses [18]. Samples treated with pronase were subsequently filtered through an 8- to 10-μm filter and the filtrate (F3) was assayed for telomerase activity qualitatively (group A5) or quantitatively (group A5-N).

Preparation of testicular or epididymal spermatozoa

Testicles (T samples) or epididymes (E samples) from group A3 were minced within SOF medium. T and E samples were filtered via an 8- to 10-μm filter and the filtrate was filtered via a Sperm Prep TM (ZBL, Lexington, Ky.) column as we previously described [29]. After a 5-minute filtration T and E filtrates were collected and underwent a centrifugation over a two-layer Percoll density gradient as we previously described [29]. The T pellet and E pellet were collected and processed for qualitative telomerase assay.

Telemerase assay (qualitative assay for telomerase activity)

Telomerase activity was qualitatively assayed in S1, S2, F1, and F2 samples of groups A1, A2, B1, C1, and D and F3 samples from group A5. In addition, it was evaluated in the testicular tissue of groups A4, C2, A6, and A7. Furthermore, qualitative telomerase assay was applied in T and E samples of group A3. Samples for qualitative telomerase assay were immediately placed in liquid nitrogen and stored at −80°C until they were subjected to the telomeric repeat amplification protocol (TRAP assay) [11, 12]. TRAP assay was performed by using the Oncor $TRAP_{EZE}$ Telomerase Detection Kit (Oncor, Gaithersburg, Md.). In brief, lysates were prepared by powdering frozen samples, followed by homogenization in 200 μl of ice-cold lysis buffer (10 mM TRIS, HCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol), and incubation on ice for 30 minutes. At the end of the incubation period, the lysates were centrifuged at 10 000 *g* at 4°C for 20 minutes and the supernatant and precipitate were rapidly frozen and stored at −80°C. The protein concentration of the supernatant was determined by the Bradford assay (Bio-Rad). Extracts equivalent to 2 μg protein were assayed in 50 μl of reaction mixture containing 20 mM TRIS, HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 μM dNTPs, TS primer, Primer Mix, and 2 units of Taq DNA polymerase (TaKaRa, Tokyo, Japan). Following a 30-minute-incubation period at 30°C to allow for telomerase-mediated extension of the TS primer, the reaction mixture was heated at 94°C for 45 seconds and then subjected to 30 polymerase chain reaction (PCR) cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. The PCR product was then electrophorosed on a 10% polyacrylamide gel.

We classified a sample as telomerase activity positive when the telomerase specific 6-bp DNA ladder was observed [10, 28]. The specificity of telomerase signals was confirmed by the observation that telomerase activity ladders were abolished by the pretreatment of extracts with RNAse.

Observation of fractions of selected cells

To confirm that our methodology was sufficient to isolate specific types of spermatogenic cells, cellular fractions from F1, S1, F2, and S2 of groups A1, A1-N, A2, B1, B1-N, C1, C1-N, and D were observed via an inverted microscope (IM; Nomarski lens; Olympus IX-70, Tokyo, Japan) computer-assisted system (CAS) [31] and a confocal scanning laser microscope (CSLM)-CAS [25]. Furthermore, minced testicular tissue from groups A4, A6, A7, and C2 was observed via IM-CAS and CSLM-CAS. Computer-assisted microscopy allows the characterization of mouse round spermatogenic cells according to the morphometric criteria proposed by Kimura and Yanagimachi [13, 14]. As secondary spermatocytes and round spermatids were characterized cells of diameters equal to 13–15 μm and 9–11 μm, respectively [13, 14]. In addition, mouse round spermatids demonstrate a dark or bright spot (acrosomal granulae) during observation via IM-CAS [18, 13] (Fig. 1). In contrast, secondary spermatocytes do not expose the above spot. Futhermore, CSLM-CAS can reveal the acrosomal cap recognizing with very high accuracy round spermatids [31, 23] (Fig. 2). As spermatogonia/primary spermatocytes were considered cells of diameter equal to 17–25 μm [13, 14] (Figs. 3, 4), primary spermatocytes were calculated together with spermatogonia cells because there are no strict morphometric criteria differentiating them [13, 14]. Fractions from T and E samples of group A3 were observed via an IM-CAS (Figs. 5, 6). F3 samples from groups A5 and A5-N were observed via both CSLM-CAS and IM-CAS (Figs. 1, 2). Specific types of spermatogenic cells were recorded (Figs. 7, 8).

RTA (quantitative assay for telomerase activity)

Testicular RTA was calculated in groups A4, A6, A7, and C2. Testicular tissue was minced into SOF medium and samples of various numbers of cells were processed for the previously described qualitative telomerase assay. We determined the RTA of a given testicular fragment by estimating the number of cells necessary for demonstration of a telomerase activity ladder of equivalent intensity to that obtained using a positive control sample (1000 human uterine cervical cancer cells) [10]. Thus, when the number of cells from a given testicular sample required to obtain a telomerase ladder of equivalent intensity to that of the positive control was 10 000 cells, the RTA of that sample was considered as $100 \times 1000/10\,000 = 10(\%)$ [10]. To find out the number of cells in a testicular sample appropriate for calculation of RTA, we assessed telomerase activity by gradually increasing the number of cells of that sample until the telomerase ladder of a specific number of cells was of the same intensity as that of the positive control. That specific cell number in the evaluated sample was used for assessment of RTA according to the above mathematic formula.

SQTA (highly sensitive quantitative telomerase assay) in testicular tissue

SQTA was evaluated according to Hisatomi and co-workers [9]. Briefly, samples for SQTA were treated as described above for the qualitative telomerase assay. Following PCR, the products were diluted with an equal volume of formamide dye solution, heated at 94°C for 5 minutes, and applied (5 μl/lane) to a 10% denaturing gel containing 6 M urea fitted to an automated DNA sequencer (ALF red DNA Sequencer, Pharmacia Biotech, Uppsala, Sweden). During electrophoresis at 45 W, the temperature of the gel was kept at 45°C. The data from the ALF red DNA Sequencer were collected and analyzed automatically by Fragment Manager v1.1 (Pharmacia Biotech, Osaka, Japan). Each peak was quantified in terms of size, peak height, and peak area. Finally the quantification of telomerase activity was carried out by the following formula [9]:

$$\frac{[\text{measured total area of telomerase activity (50 bp, 56 bp, 62 bp, 68 bp}\ldots)]/[\text{measured area of internal control (36 bp)}]}{[\text{measured total area of telomerase activity (50 bp, 56 bp}\ldots 68) \text{ in positive control}]/[\text{measured area of internal control (36 bp) in positive control}]} \times 100 = \text{TPG (total product generated) units}/\mu\text{g protein}$$

SQTA in germ cell subpopulations

SQTA was measured as above in the recovered S1, S2, and F2 fractions of groups A1-N and B1-N. Furthermore, SQTA was evaluated in a part of the F1 fraction prior to the second filtration in the above groups. SQTA was also evaluated in S1 samples of the group C1-N and F3 samples of the group A5-N.

Results

Both IM-CAS and CSLM-CAS showed that more than 79% of the cells in S1 samples of all animals of groups A1, A2 and B1 were spermatogonia plus primary spermatocytes plus secondary spermatocytes (Table 1). Remaining cells were mainly Sertoli cell fragments. Within group D, S1, F1, and S2 samples included mainly Sertoli cell fragments and leukocytes. In groups A1 and A2, F1 samples contained secondary spermatocytes plus spermatids plus spermatozoa (total percentage of these spermatogenic cells $>86\%$ in each animal). F1 samples of group B1 contained secondary spermatocytes plus round spermatids (total percentage $>83\%$ in each animal). Within group C1, F1 samples contained no spermatogenic cells. S2 samples of groups A1 and A2 contained secondary spermatocytes plus round spermatids plus elongating spermatids plus elongated spermatids plus spermatozoa (total percentage $>86\%$ in each animal). S2 samples of group B1 contained secondary spermatocytes plus round spermatids (total percentage $>85\%$ in each animal). F2 samples in groups A1 and A2 contained round spermatids plus elongating spermatids plus elongated spermatids plus spermatozoa (total percentage $>84\%$ in each animal; Table 1), whereas, in group B1 they contained mainly round spermatids (total percentage $>90\%$ in each animal). F3

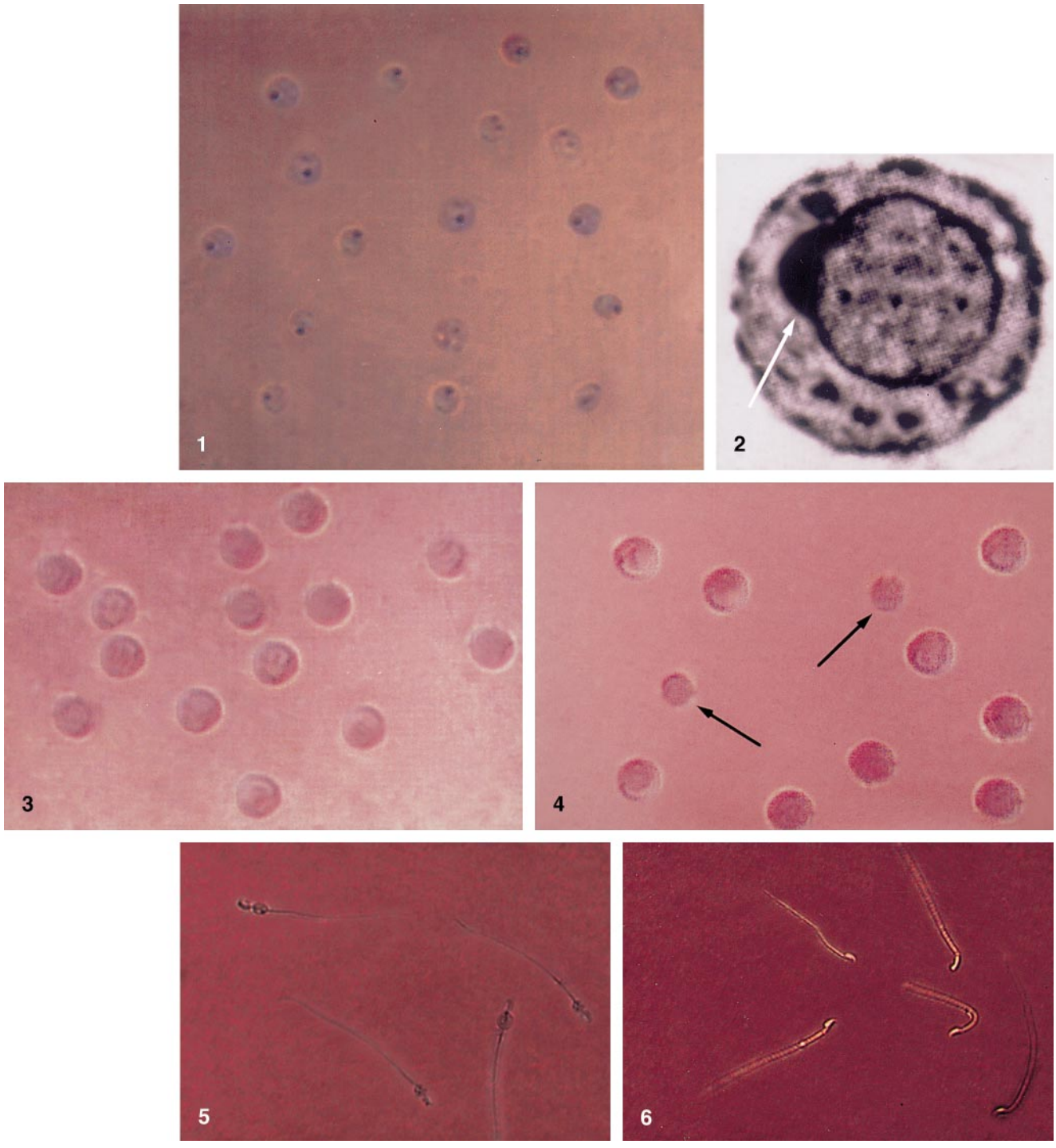


Fig. 1 Observation of a fraction of round spermatids (F3 of group A5) via inverted microscope (×200) computer-assisted system (×2). Round spermatids are characterized by a black spot (acrosomal granule)

Fig. 2 Observation of a round spermatid via confocal scanning laser microscope (×3600) computer-assisted system (×2). An acrosomal granule (*arrow*) attached to the nucleus is observable

Fig. 3 Observation of a fraction of spermatogonia plus primary spermatocytes (S1 of group C1) via inverted microscope (×200) computer-assisted system (×2.25)

Fig. 4 Observation of a fraction of spermatogonia/primary spermatocytes plus secondary spermatocytes (S1 fraction of group A1) via inverted microscope (×200) computer-assisted system (×2.5). Secondary spermatocytes are indicated by *arrows*

Fig. 5 Observation of a fraction of testicular spermatozoa (T sample of group A3) via inverted microscope (×200). Cytoplasmic droplets are present at the neck regions

Fig. 6 Observation of a fraction of epididymal spermatozoa (E sample of group A3) via inverted microscope (×200)

samples in group A5 contained round spermatids (total percentage >93% in each animal). T samples from group A3 consisted of testicular spermatozoa (total percentage >89% of the cells in each animal), whereas E samples contained epididymal spermatozoa (total percentage >96% in each animal). The mean value and the standard deviation of the percentage of each spermatogenic cell constituting the above samples are shown in Table 1.

To isolate subpopulations of spermatogenic cells, minced testicular samples were initially filtered via a filter of 11–13 μm diameter. This filter removed mainly mouse spermatogonia plus primary spermatocytes and subsequently S1 samples were rich in spermatogonia/primary spermatocytes. F1 samples in healthy mature animals contained secondary spermatocytes which had not been removed by the first filter, spermatids, and spermatozoa. The second filter (8–10 μm diameter) removed the vast majority of secondary spermatocytes and a minor population of spermatids from the F1 samples. The great majority of spermatids from F1 samples passed through the second filter. Thus, S2 samples contained a relatively large proportion of secondary spermatocytes.

Hematoxylin and eosin revealed all types of spermatogenic cells in histologic sections of group A1 and A2 (Fig. 9). However, it recognized spermatogonia plus primary spermatocytes only in group C1 (Fig. 10). Spermatogonia, primary spermatocytes, and a limited number of round spermatids were demonstrated in group B1. No spermatogenic cells were found in histologic sections from testicles of group D.

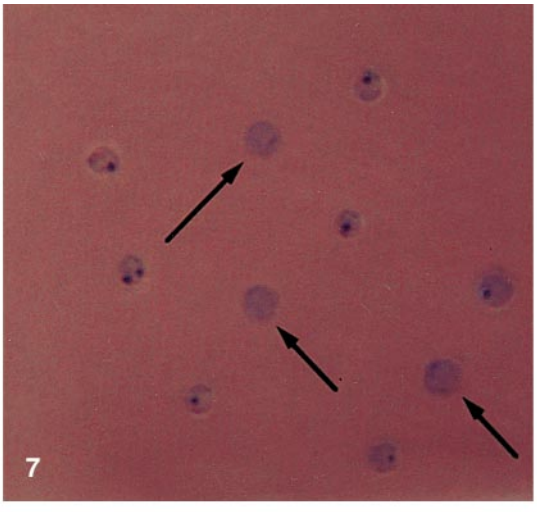
Relative telomerase activity was not significantly different ($P > 0.05$; analysis of variance) among groups A4, A6, A7 and C2 (Table 2). Furthermore differences in testicular tissue SQTA outcome among groups A8,

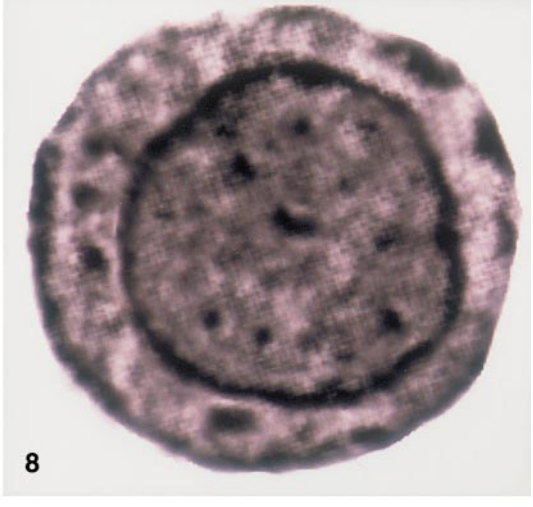
Fig. 7 Observation of a fraction of secondary spermatocytes (*arrows*) plus round spermatids (S2 of group B1) via inverted microscope (×200) computer-assisted system (×1). Round spermatids show as a black spot

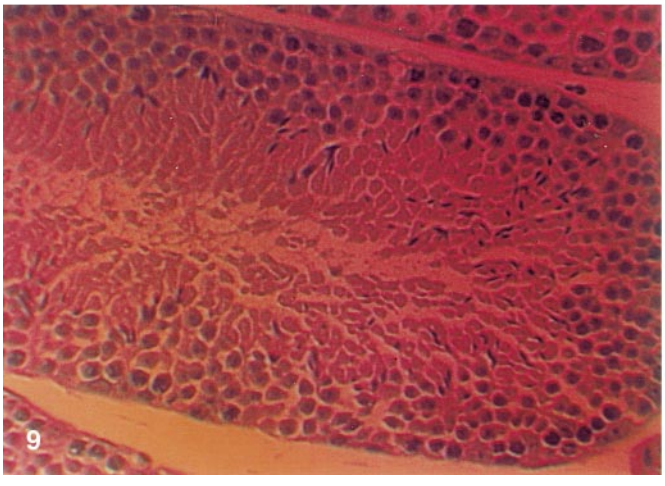
Fig. 8 Observation of a secondary spermatocyte via confocal scanning laser microscope (×3600) computer-assisted system (×1.25)

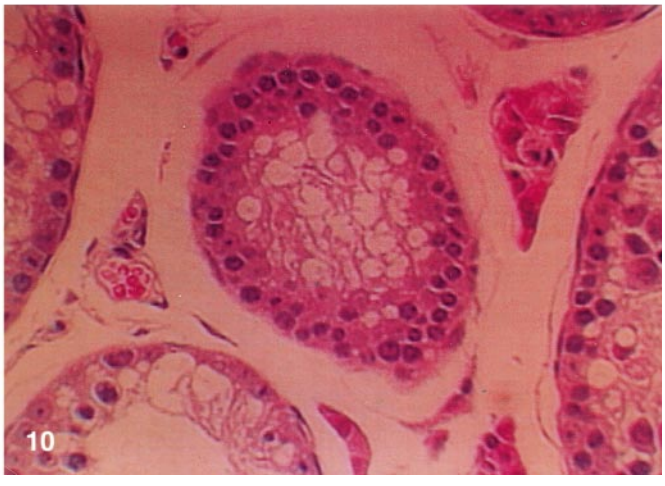
Fig. 9 Hematoxylin and eosin staining of testicular tissue of group A1 animals showing a normal spermatogenesis

Fig. 10 Hematoxylin and eosin staining of testicular tissue of group C1 animals showing spermatogenic arrest at the primary spermatocyte stage









A9, B2, and C3 were not significant ($P > 0.05$; analysis of variance; Table 3).

Differences in the SQTA profile of S1 fraction among groups A1-N, B1-N, and C1-N were not significant ($P > 0.05$; analysis of variance). In contrast, within group A1-N and within group B1-N the SQTA outcome was significantly larger in the S1 fraction than in F1, S2, or F2 fractions (analysis of variance followed by Duncan's multiple range test; $P < 0.05$). Furthermore, SQTA outcome in the S1 fraction of 8-week-old healthy mice (group A1-N) was significantly larger than the SQTA outcome in the F3 fraction of another group of healthy mice of the same age (group A5-N; Wilcoxon test; $P < 0.05$). In addition, SQTA outcome was significantly smaller in minced testicular tissue of groups B2 or A9 than in S1 fractions of the same age groups B1-N or A1-N, respectively (Wilcoxon's test; $P < 0.05$; Tables 3, 4). In contrast, there was no significant difference in SQTA outcome between minced testicular tissue of the cryptorchid group C3 and S1 fraction of the same age-cryptorchid group C1-N ($P > 0.05$; Wilcoxon's test; Tables 3, 4). Difference in SQTA outcome between F3 fraction of group A5-N and F2 fraction of group B1-N was not significant ($P > 0.05$; Wilcoxon's test). SQTA outcome in minced testicular tissue of the cryptorchid group B1-N ($P < 0.05$; Wilcoxon's test; groups B1-N and B2 were of the same age). Furthermore, SQTA outcome in minced testicular tissue of group A9 was significantly larger than the SQTA result in F3 fraction of group A5-N ($P < 0.05$; Wilcoxon's test; groups A9 and A5-N include healthy mice of the same age).

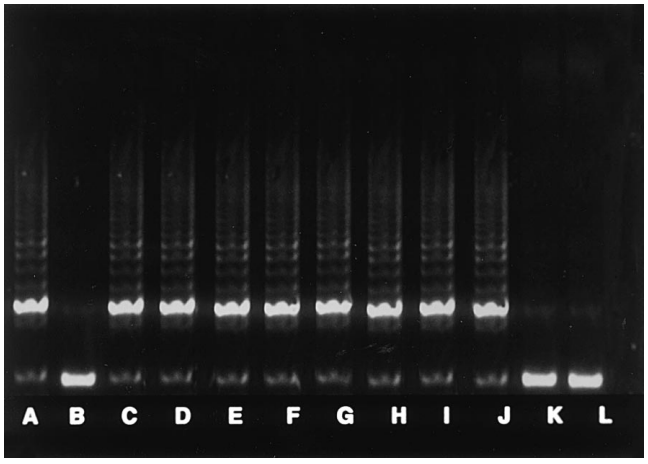


Fig. 11 Telomerase assay of testicular tissue/fractions of spermatogenic cells. *A* positive control, *B* negative control, *C* S1 sample in group A1, *D* F1 sample in group A1, *E* S1 sample in group C1, *F* group A4, *G* F3 sample of group A5, *H* group C2, *I* group A6, *J* group A7, *K* T-sample in group A3, *L* E-sample in group A3

Table 1 Expression of telomerase in selected subpopulations of mouse spermatogenic cells

Group	*n*	Sample	Main spermatogenic cells (mean ± SD; %)	TA
A1	9	S1	[SPRG + PS](84 ± 4), SS(3 ± 1)	+
		F1	SS(4 ± 2), S(72 ± 6), SZ(15 ± 5)	+
		S2	SS(30 ± 5), S(50 ± 7), SZ(11 ± 4)	+
		F2	S(75 ± 8), SZ(16 ± 6)	+
A2	9	S1	[SPRG + PS](86 ± 4), SS(2 ± 1)	+
		F1	SS(3 ± 1), S(69 ± 8), SZ(18 ± 7)	+
		S2	SS(28 ± 5), S(52 ± 6), SZ(10 ± 3)	+
		F2	S(73 ± 7), SZ(14 ± 5)	+
B1	12	S1	[SPRG + PS](88 ± 5), SS(3 ± 1)	+
		F1	SS(5 ± 2), RS(83 ± 4)	+
		S2	SS(28 ± 7), RS(64 ± 9)	+
		F2	RS(95 ± 2)	+
C1	9	S1	[SPRG + PS](89 ± 5)	+
		F1	No	–
D	9	S1	No	–
A3	5	T samples	SZ(94 ± 2)	–
		E samples	SZ(97 ± 1)	–
A4	8	Testis	[SPRG + PS](65 ± 6), SS(2 ± 1), S(18 ± 5), SZ(4 ± 1)	+
A5	5	F3	RS(96 ± 1)	+
C2	8	Testis	[SPRG + PS](95 ± 2)	+
A6	8	Testis	[SPRG + PS](93 ± 3)	+
A7	8	Testis	[SPRG + PS](81 ± 4), SS(1 ± 1), RS(6 ± 2)	+
A1-N	10	S1	[SPRG + PS](88 ± 5), SS(4 ± 1)	
		F1	SS(5 ± 2), RS(75 ± 7), SZ(14 ± 5)	
		S2	SS(28 ± 6), S(55 ± 8), SZ(12 ± 4)	
		F2	S(79 ± 7), SZ(15 ± 5)	
B1-N	10	S1	[SPRG + PS](92 ± 2), SS(4 ± 1)	
		F1	SS(7 ± 2), RS(80 ± 5)	
		S2	SS(31 ± 8), RS(62 ± 10)	
		F2	RS(93 ± 2)	
C1-N	10	S1	[SPRG + PS](92 ± 3)	
A5-N	10	F3	RS(94 ± 2)	
A9	8	Testis	[SPRG + PS](61 ± 7), SS(3 ± 1), S(21 ± 6), SZ(6 ± 2)	+
B2	8	Testis	[SPRG + PS](74 ± 5), SS(4 ± 2), RS(14 ± 5)	+

SPRG spermatogonia, *PS* primary spermatocytes, *SS* secondary spermatocytes, *S* spermatids (round, elongating, and elongated spermatids), *RS* round spermatids, *SZ* spermatozoa, *TA* telomerase activity, *n* number of animals in each group

Table 2 Relative telomerase activity (RTA) in the mouse testis

Group	Testis	*n*	Spermatogenic cells	TA	RTA (%)
A4	Healthy; 10-week-old	8	SPRG, PS, SS, S, SZ	+	8.8 ± 1.8
C2	Cryptorchid; 10-week-old	8	SPRG, PS	+	8.0 ± 1.7
A6	Healthy; 17-day-old	8	SPRG, PS	+	7.2 ± 2.7
A7	Healthy; 22-day-old	8	SPRG, PS, SS, RS	+	7.0 ± 2.1

RTA (%) = 100 × A/B; A = 1000 cells of the positive control, B = number of cells of the evaluated sample required to obtain a telomerase ladder of the same intensity to that obtained using 1000 cells of the positive control
Values are expressed as means ± SD

Table 3 Outcome of highly sensitive quantitative telomerase assay (SQTA) in the mouse testis

Group	*n*	Testis	Spermatogenic cells	SQTA (total product generated units/μg protein)
A8	8	Healthy; 10 weeks old	SPRG, PS, SS, S, SZ	63.31 ± 10.81
A9	8	Healthy; 8 weeks old	SPRG, PS, SS, S, SZ	60.03 ± 8.41
B2	8	Cryptorchid; 8 weeks old	SPRG, PS, SS, RS	57.13 ± 9.93
C3	8	Cryptorchid; 10 weeks old	SPRG, PS	55.27 ± 9.87

Values are expressed as means ± SD
No significant differences were found in mean SQTA value among groups A8, A9, B2, and C3 ($P > 0.05$)

Discussion

Telomerase activity has been previously demonstrated in the mouse and rat testis [6, 21]. However, the specific cell types expressing telomerase activity in the mouse testis are unknown. We attempted to investigate whether the testicular tissue telomerase activity is due to telomerase expression in all cells or in specific cellular populations. To address this issue five methods were used: (a) testicular tissue from healthy mature mice was minced and filtered through specific filters excluding primary spermatocytes (11- to 13-μm diameter filter) or secondary spermatocytes (8- to 10-μm diameter filter); thus telomerase activity was qualitatively assessed in cellular fractions containing selected subpopulations of spermatogenic cells; (b) spermatogenic arrest at the primary spermatocyte stage (group C1) or round spermatid stage (group B1) was induced and purified cellular subpopulations were prepared and processed for qualitative telomerase assay; (c) telomerase activity was qualitatively assessed in testicular tissue of prepubertal animals with spermatogenic arrest at the primary spermatocyte stage (group A6) or round spermatid stage (group A7); (d) RTA or SQTA were employed to quantify telomerase activity in testicular tissue with specific types of spermatogenic cells (groups A4, A6, A7, and C2 or A8, A9, B2, and C3, respectively); (e) SQTA was applied to specific germ cell subpopulations in groups A1-N, B1-N, C1-N, and A5-N.

CSLM-CAS and IM-CAS proved that our methodology for preparation of fractions of specific spermatogenic cells resulted in highly purified spermatogenic cellular subpopulations. All the above five experimental approaches indicated that samples of spermatogonia/primary spermatocytes, or secondary spermatocytes plus round spermatids or round spermatids alone are positive for telomerase activity. In contrast, testicular and epididymal spermatozoa are negative for telomerase activity. It appears that telomerase activity is inhibited during the transformation of the round spermatid to spermatozoon. The absence of telomerase activity in spermatozoa is consistent with a previous study showing that transcription and translation do not occur in spermatozoa and that spermatozoal DNA polymerase is not active [8]. Telomerase is expected to remain active in primary spermatocytes and secondary spermatocytes to ensure the transmission of full-length chromosomes to the round spermatid and subsequently to the spermatozoon. The current study is consistent with the identification of telomerase activity in rat pachytene spermatocytes and round spermatids [6]. Both the current study and a previous study [6] suggest that telomerase may play an important role in lengthening telomere during meiotic divisions. However, it is difficult to explain the biological role of telomerase expression at the round spermatid stage considering that this cell does not undergo further division.

Ooplasmic injections of round spermatids are currently applied for the treatment of non-obstructive azoospermia [1, 4, 24, 25, 26, 27]. In the natural human fertilization process the telomerase-negative nucleus of a spermatozoon [28] and the telomerase-negative nucleus of an oocyte [28] participate in syngamy. Considering that telomerase expression has been implicated in tumorgenesis [11], the presence of telomerase activity in round spermatids and subsequently in the zygote (prior to its first mitotic division) may imply genetic risks for the embryo or fetus. On the other hand, telomerase expression is expected in embryos, since the telomerase

hypothesis [6] suggests that telomerase is active in embryonic cells and that this activity decreases during differentiation in a tissue- and cell-specific manner. To illustrate this subject additional studies are necessary on telomerase activity and telomere length during the early embryonic development.

Spermatogenic arrest at the primary spermatocyte stage or round spermatid stage occurs during prepubertal development or primary testicular damage. Since TRAP assay is a qualitative and not a quantitative assay, we calculated the testicular tissue RTA to compare the telomerase activity between a healthy testis and a testis with spermatogenic arrest at the primary spermatocyte stage or the round spermatid stage. Non-significantly different RTA values were found. To investigate this issue further, we evaluated testicular tissue telomerase activity quantitatively by applying the SQTA, for the first time in the international literature, in healthy testicles and testicles with spermatogenic arrest. The latter assay is highly sensitive and is considered the gold standard assay to assess quantitatively tissue telomerase activity [9, 15, 19]. Differences in the SQTA outcome were not significant (Table 3). It appears that (a) the stimulus for disruption of spermatogenesis (i.e., temperature in cryptorchid mice) does not influence significantly the expression of telomerase in the remaining spermatogenic cellular populations; (b) the elimination of round spermatids does not result in significant quantitative changes in testicular tissue telomerase activity; and (c) the absence of elongating and elongated spermatids (i.e., groups A7 and B2) does not lead to significant quantitative alterations in tissue telomerase activity. Thus, although telomerase activity was demonstrated in a highly purified fraction of round spermatids (F3 samples of group A5), our findings tend to suggest that spermatogonia and primary spermatocytes represent the main source of telomerase activity in the testis. This is further supported by the lack of telomerase activity in testes of animals with Sertoli cell only pathophysiology.

We compared the SQTA profiles of highly purified spermatogenic cell subpopulations (Table 4). These studies further confirmed our theses first that spermatogonia/primary spermatocytes represent the cellular subpopulation with the largest telomerase activity in the testis. This is supported by the finding that the SQTA outcome was significantly larger in S1 fraction than in F1, S2, and F2 fractions within the group A1-N and within the group B1-N. It is also supported by the finding that the SQTA outcome was significantly larger in highly purified spermatogonia/primary spermatocytes plus secondary spermatocytes of healthy mice (S1 fraction of group A1-N) than in a highly purified round spermatid fraction of another group of healthy mice of the same age (F3 fraction of group A5-N). Second, that the expression of telomerase does not alter significantly in a specific germ cell subpopulation (the subpopulation of spermatogonia/spermatocytes or the subpopulation of round spermatids) in cryptorchid animals. This is supported by the absence of a significant difference in SQTA outcome in spermatogonia/primary spermatocytes plus secondary spermatocytes (fraction S1) between groups A1-N (healthy animals) and B1-N (cryptorchid animals). The latter thesis is additionally supported by the absence of a significant difference in SQTA outcome in purified round spermatids between healthy 8-week-old mice (fraction F3 of group A5-N) and cryptorchid 8-week-old mice (fraction F2 of group B1-N; see Results).

Considering that the minced testicular tissue processed for SQTA in group B2 contained spermatogonia/spermatocytes and round spermatids, whereas the F2 fraction of a group of cryptorchid animals of the same age (group B1-N) contained highly purified round spermatids, the significantly larger SQTA outcome in testicular tissue of B2 than in F2 fraction of group B1-N may be attributable to the presence of spermatogonia/primary spermatocytes in the former samples. The latter cells have been proven to demonstrate greater telomerase activity than round spermatids. Similarly the significantly larger SQTA outcome in the minced testicular tissue of group A9 than the SQTA profile of purified round spermatids of same age healthy animals (fraction F3 of group A5-N) may be attributable to the presence of spermatogonia/primary spermatocytes in the testicular tissue of animals of group A9. In contrast, the SQTA outcome was significantly smaller in (a) minced testicular tissue of cryptorchid mice (group B2) than in purified

Table 4 Outcome of SQTA (total product generated units/μg protein) in selected fractions of spermatogenic cells

Group	*n*	Age (weeks)	SQTA in specific spermatogenic cell subpopulations				
			S1	F1	S2	F2	F3
A1-N	10	8	74.06 ± 9.18[a]	38.22 ± 11.61[b]	47.98 ± 13.91[b]	34.96 ± 13.97[b]	
B1-N	10	8	72.94 ± 8.24[a]	40.65 ± 10.11[b]	49.05 ± 13.72[b]	38.08 ± 10.61[b]	
C1-N	10	10	63.92 ± 14.81[a]				
A5-N	10	8					45.06 ± 8.53

Animals in groups A1-N, B1-N, C1-N, and A5-N were of the same age/underwent the same treatment as animals in groups A1, B1, C1, and A5, respectively (see Table 1)

Values are expressed as means ± SD

[a, b] Within each line and within each column values not sharing the same superscript [a] or [b] are significantly different ($P < 0.05$)

The difference in SQTA outcome between F2 fraction of group B1-N (highly purified round spermatids) and F3 fraction of group A5-N (highly purified round spermatids) was not significant ($P > 0.05$)

spermatogonia/spermatocytes of cryptorchid animals of the same age (S1 fraction of group B1-N), and (b) minced testicular tissue of healthy mice (group A9) than in purified spermatogonia/spermatocytes of healthy animals of the same age (S1 fraction of group A1-N). These last two significant differences may be due to the larger number of spermatogonia/primary spermatocytes per total cell number in the S1 fractions of groups B1-N or A1-N than in minced testicular tissue of groups B2 or A9, respectively (see Table 1). Furthermore, there was no significant difference in SQTA outcome between minced testicular tissue of cryptorchid animals (group C3) and purified spermatogonia/primary spermatocytes of a group of cryptorchid mice of the same age (fraction S1 of group C1-N). This absence of significant difference may be due to the fact that both the former and latter samples consisted of the same germ cell type (i.e., spermatogonia/primary spermatocytes).

The current study demonstrated telomerase activity in 17-day-old and 22-day-old mice. Furthermore, the mean testicular RTA in the above groups was similar to that in mature animals. A previous study using a different species of mouse (*Mus musculus*) did not detect telomerase activity in the testes of 4-week-old animals [21]. This difference may be due to the different speed of spermatogenesis between *Mus musculus* and the B6D2F1 mice we used.

The presence of telomerase activity in round germ cells and the absence of telomerase activity in subjects with Sertoli cell-only syndrome is of clinical importance in assisted reproduction programs. Recent studies [30] have shown that the qualitative telomerase assay in men with Sertoli cell-only syndrome can identify subpopulations with testicular foci of active spermatogenesis. Furthermore, it was found that men with Sertoli cell-only syndrome and SQTA profiles >42 TPG units/μg protein in a diagnostic testicular biopsy specimen may be candidates in assisted reproduction programs because there is a large probability that foci of haploid cells exist within the testicular tissue. It appears that both qualitative and quantitative telomerase assays have an important role in the diagnosis and treatment of men with Sertoli cell-only syndrome.

Our findings may suggest that primary testicular damage does not influence quantitatively the telomerase activity of the remaining primary spermatocytes or primary spermatocytes plus round spermatids. We have also shown that telomerase activity is significantly larger in isolated subpopulations of spermatogonia/primary spermatocytes than in purified fractions of round spermatids. Telomerase activity appears to be inhibited during mouse spermiogenesis. Additional studies are necessary to indicate the exact stage of the spermiogenesis at which the male gamete loses its telomerase activity.

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