stem cells (19). However, when we analyzed the injected testes 4–8 weeks after adenovirus injection we found only patchy infection in the Sertoli cells that received adenovirus at the immature stage (Fig. 1*A*). Although staining was also found in mature testes, this was similarly localized exclusively in the Sertoli cells, and we were not able to obtain clear evidence of germ cell infection (Fig. 1*B*). Testes transduced with adenovirus vector occasionally showed signs of inflammation, but spermatogenesis and expression of the *LacZ* gene were observed in all treated testes. In agreement with these results, when we microinjected EGFP-expressing adenovirus (AxCANEGFP) into mature testes, none of the 12 injected males sired transgenic progeny after mating with wild-type females. Although >100 offspring were analyzed for EGFP expression, we did not observe EGFP fluorescence (data not shown).

Infection of Spermatogonial Stem Cells *in Vitro*. We next sought to examine whether adenovirus can infect spermatogonial stem cells *in vitro*. We dissociated immature testes and cultured them in the presence of AxCANEGFP. The immature testes contain enriched populations of spermatogonial stem cells because they lack differentiated cells (18). After infection, EGFP-expressing cells were found on the next day in culture (Fig. 1 *C* and *D*). To determine whether germ cells could be infected by the adenovirus, the infected cells were analyzed by flow cytometry 2 days after infection. The analysis revealed that 13.3% of the EGFP-positive cells expressed the spermatogonia marker EpCAM (Fig. 1*I*) (20).

To further examine whether the virus can infect spermatogonial stem cells, we exposed AxCANEGFP to cultured spermatogonial stem cells or germ-line stem cells (GS cells) (21). These cells can undergo self-renewing division in the presence of glial cell line-derived neurotrophic factor *in vitro*, but they reinitiate spermatogenesis after transplantation into seminiferous tubules. After overnight infection with AxCANEGFP, most of the GS cell colonies showed EGFP fluorescence (Fig. 1 *E*, *F*, and *J*). The EGFP expression continued after several passages. Within 2–3 weeks the fluorescence was barely detectable, which suggested that the vector did not integrate stably in the genome of the GS cells (Fig. 1*L*). Although we did not find a significant effect of virus on the growth and morphology of GS cells at low virus concentrations (6×10^3 pfu/ml), exposure to higher concentrations of adenovirus had a negative effect on GS cell growth, and only 13% of the cultured cells could be recovered at 6×10^5 pfu/ml 6 days after infection (Fig. 1*K*). Adenovirus could also infect ES-like multipotent GS cells (mGS cells) (Fig. 1 *G* and *H*) (22). These results established that spermatogonia can be infected *in vitro* by adenovirus vector.

Spermatogenesis from Adenovirus-Infected Stem Cells. To establish that spermatogonial stem cells are infected by adenovirus, it is necessary to test whether infected cells can initiate and maintain long-term spermatogenesis by spermatogonial transplantation, which is the only functional assay for spermatogonial stem cells (23). However, we assumed that detecting signs of infection might be difficult in germ cell colonies because adenovirus generally remains as an episomal element and may disappear as single stem cells undergo multiple rounds of division to mature into spermatozoa (24). In agreement with this possibility, the intensity of EGFP fluorescence in the AxCANEGFP-infected GS cells decreased during passages (Fig. 1*L*), and the injection of AxCANEGFP-infected testis cells into seminiferous tubules of infertile adult recipient animals did not yield clear evidence of germ cell transduction (data not shown).

To establish a more sensitive method for detection of viral gene transduction, we took advantage of the Cre recombinase system (Fig. 2) (25). In this experiment, testis cells used for infection were collected from ROSA26 Cre reporter (R26R) mice that were 5–10 days old. At this stage, spermatogonial stem cells are enriched in the testis because of the absence of differentiated cells. The testis cells were exposed overnight to Cre-expressing adenovirus (AxCANCre) *in vitro* (26), and the

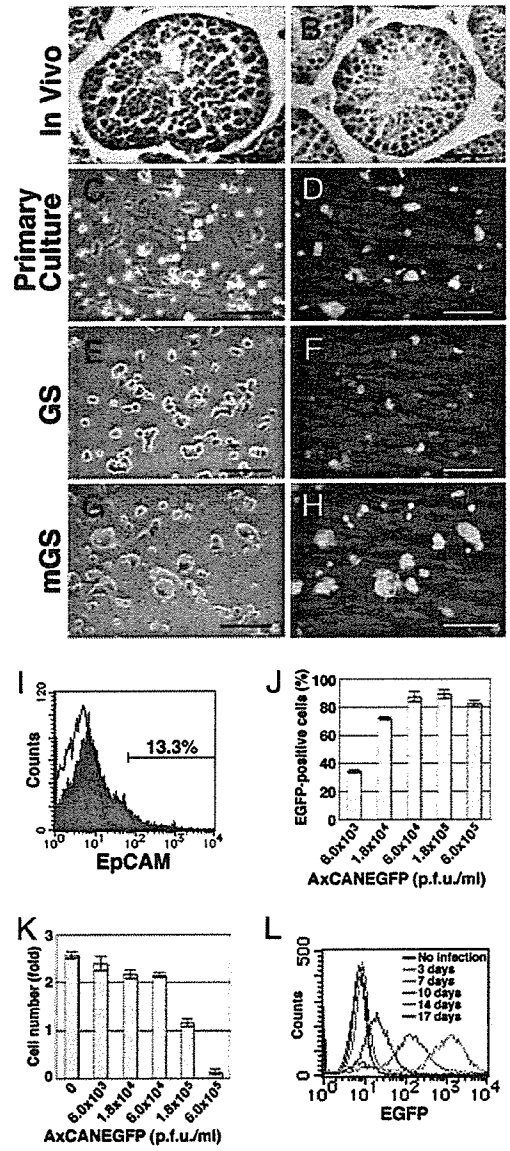

Fig. 1. Adenovirus-mediated gene transfer into male germ cells. (*A* and *B*) Histological appearance of testes injected with AxCANLacZ at 7 days (*A*) or 4 weeks (*B*) after birth. Whole mounts of testes were stained 1 month after virus injection. (*C–H*) *In vitro* infection of immature testis cells (*C* and *D*), GS cells (*E* and *F*), and mGS cells (*G* and *H*) by AxCANEGFP. The cells were exposed to adenovirus overnight at 2.5×10^5 pfu/ml (*C* and *D*) or 1.8×10^5 pfu/ml (*E–H*), and EGFP fluorescence was examined 6 h (*D*) or 1 day (*F* and *H*) after infection. (*I*) Flow-cytometric analysis of immature testis cells 2 days after transduction of AxCANEGFP at 2.5×10^5 pfu/ml. EpCAM-positive spermatogonia cells showed EGFP fluorescence. Black line, control Ig; red line, specific antibody. (*J*) Flow-cytometric analysis of GS cells 3 days after transduction of AxCANEGFP. The values are mean ± SEM ($n = 3$). (*K*) Increase in GS cell number after adenovirus infection. After overnight infection, GS cells were cultured for 6 days. The values are mean ± SEM ($n = 4$). Although no significant difference in cell number was found at 6.0×10^3 pfu/ml, GS cell growth was inhibited at higher virus concentrations ($P < 0.05$ by *t* test). (*L*) The intensity of EGFP signals decreased during a 17-day culture. The cells were infected at 2.5×10^5 pfu/ml and passaged twice during this period. (Scale bars: 50 μm for *A* and *B* and 100 μm for *C–H*.)

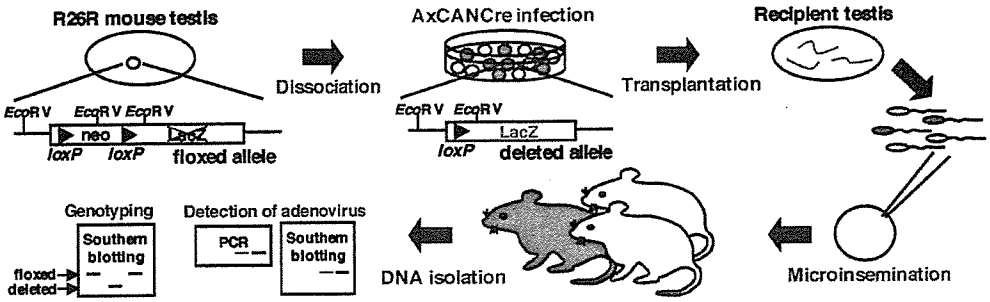

Fig. 2. Diagram of the experimental procedure. Testis cells from donor R26R mice were dissociated by trypsin digestion and infected *in vitro* by AxCANCre adenovirus. Cre-mediated recombination removed the neo cassette, and LacZ gene expression was initiated under the ROSA26 ubiquitous promoter. The infected cells were transplanted into infertile recipient testes. At 20 weeks after transplantation, recipient testes were mechanically dissociated, and spermatogenic cells were microinjected into oocytes to produce offspring. DNA from the offspring was analyzed by Southern blotting and PCR for integration of adenovirus.

infected cells were collected by trypsin digestion on the next day for transplantation into the seminiferous tubules of histocompatible recipient mice that were 5–10 days old, allowing the efficient colonization of donor cells (18). Successful Cre-mediated recombination in infected cells would result in the deletion of DNA sequences that are flanked by *loxP* sequences. Because ROSA26 promoter is active during spermatogenesis, the infected cells start to express *LacZ* gene after Cre-mediated recombination, which can be readily detected by X-Gal staining (27).

Four separate experiments were performed, and a total of 17 testes in 14 recipient animals were microinjected with the cultured cells. PCR and Southern blot analyses of the cultured cells revealed that 49–76% of the cells underwent Cre-mediated recombination after overnight incubation with Cre-expressing adenovirus (Fig. 3). This deletion occurred only when the cells were exposed to AxCANCre (Fig. 3*B*), and sequence analysis of the cultured cells confirmed that *loxP* regions were maintained after culture without the presence of AxCANCre (data not shown). Three months after transplantation, testes were recovered from some of the recipients and stained for LacZ activity. LacZ-expressing colonies were found in all recipient testes, indicating that Cre recombinase was successfully expressed and induced recombination in spermatogonial stem cells (Fig. 4*A*). Histological analysis of the recipient testes showed normal-appearing spermatogenesis, and mature spermatozoa could be found in the germ cell colony (Fig. 4*B*). LacZ-expressing round and elongated spermatids were also observed. Because spermatogonial stem cells are the only cell type that can establish complete spermatogenesis after transplantation, these results show that spermatogonial stem cells infected with adenovirus vector could induce normal spermatogenesis.

Lack of Adenovirus Integration in Offspring from Infected Stem Cells. To determine whether the germ cells from the infected cells are fertile, we used *in vitro* microinsemination, a technique commonly used to produce offspring in animals and humans (28, 29). Testes or epididymides were collected from three different recipients 7 months after transplantation of adenovirus-infected donor cells. Seminiferous tubules or epididymis were dissected by fine forceps and mechanically dissociated by repeated pipetting. The recovered round spermatids and elongated spermatids and spermatozoa were microinjected into oocytes from C57BL/6 (B6) × DBA/2 F_1 (BDF1) females. After 24 h of culture, 23 of 123 (19%) of the embryos developed to the two-cell stage, and they were transferred into the oviducts of pseudopregnant ICR females. Of the 85 embryos transferred, 46 (54%) implanted in the uteri and 21 offspring were born (Table 1). Overall, offspring were obtained from three of four recipient males that were used in microinsemination, and PCR and Southern blot analyses revealed that successful recombination occurred in all 14 offspring that carried ROSA floxed allele (Fig. 4 *D* and *E*). Nine offspring, six males and three females, grew up to be normal fertile adults. When we examined the expression of the *LacZ* gene, three of the nine offspring showed X-Gal staining in skin

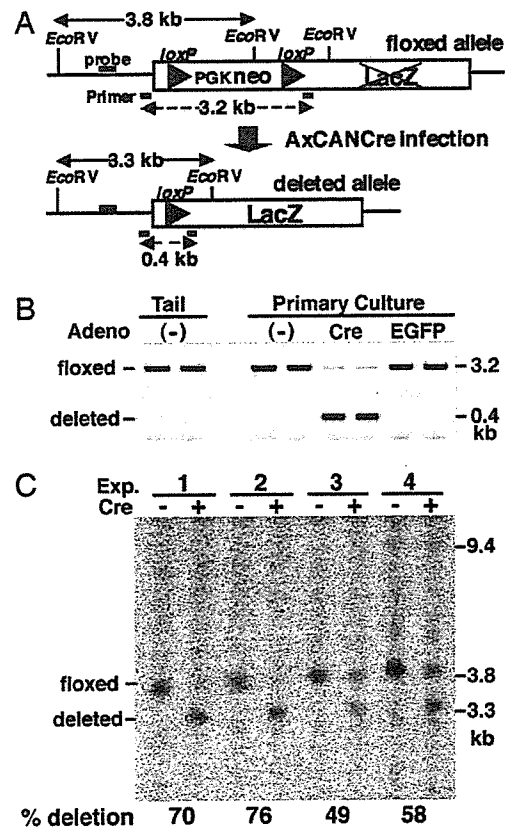

Fig. 3. Deletion of floxed sequence in ROSA26 locus. (*A*) Diagram of the experimental design to detect deletion of floxed sequence by PCR and Southern blot analyses. (*B*) PCR analysis of the AxCANCre-mediated deletion by *in vitro* infection of immature testis cells. Deletion was detected only when the cultured cells were exposed to AxCANCre. Deletion did not occur when AxCANEGFP was used for infection. (*C*) Deletion efficiency of ROSA26 locus. Genomic DNA of infected cells was digested with EcoRV and hybridized with a ROSA26-specific probe (see *Materials and Methods*). Levels of percentage deletion, estimated by the intensity of each band, are indicated below.

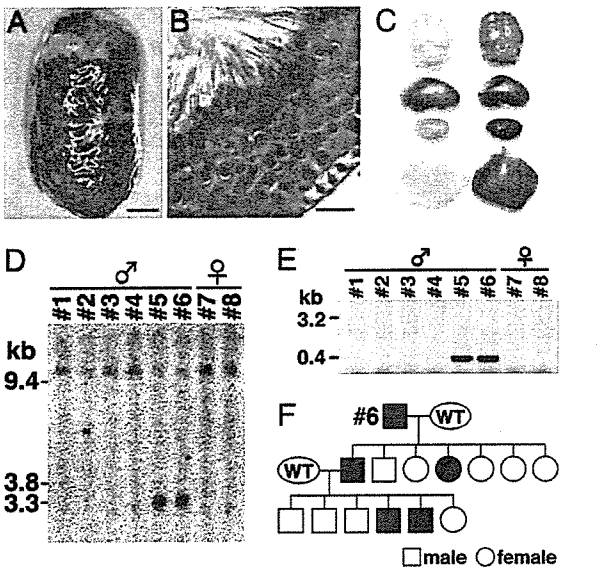

Fig. 4. LacZ expression after Cre recombination. (*A*) Macroscopic appearance of a recipient testis that received Cre-infected testis cells 3 months after transplantation. Blue tubules represent colonization of donor stem cells that were infected by AxCANCre adenovirus. (*B*) Histological appearance of a recipient testis. Note complete spermatogenesis. LacZ expression was found in all spermatogenic cells. (*C*) Macroscopic appearance of X-Gal-stained brain, kidney, testis, and liver of F_1 offspring from AxCANCre-infected spermatogonial stem cells. Note ubiquitous LacZ expression. (*D*) Southern blot analysis of EcoRV-digested tail genomic DNA from mature F_1 offspring hybridized with a ROSA26-specific probe. Two offspring showed Cre-mediated recombination. (*E*) PCR analysis of the deletion. The deletion of floxed sequence was confirmed by PCR detection method, as shown in Fig. 3*A*. (*F*) Pedigree of an F_1 male demonstrating transmission of parental genotype for three generations. Solid symbols indicate LacZ expression in these progeny. (Scale bars: 1 mm for *A* and 20 μm for *B*.)

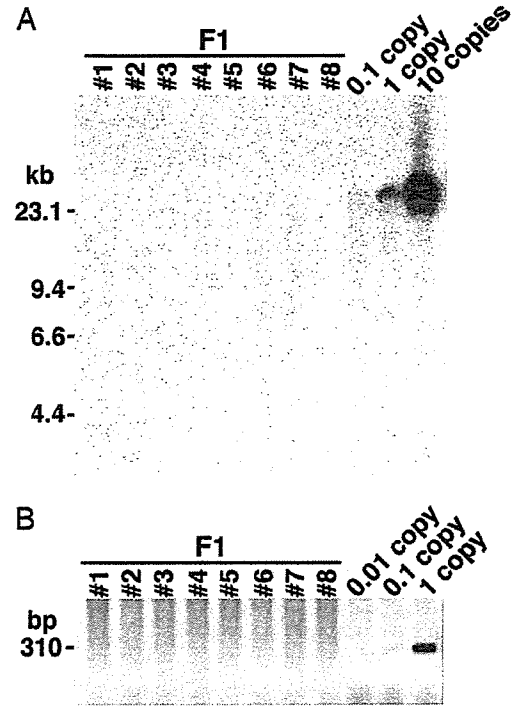

Fig. 5. Lack of adenovirus integration in the mature F_1 offspring. (*A*) Southern blot analysis of F_1 DNA samples hybridized with a probe specific for adenoviral sequences (see *Materials and Methods*). Controls represent viral DNA in amounts equivalent to 0.1, 1, and 10 copies of viral DNA per diploid genome. (*B*) PCR analysis of the F_1 DNA samples. The adenovirus-specific 310-bp fragment was amplified from the F_1 DNA samples. Controls containing 0.15 μg of normal mouse DNA were spiked with viral DNA representing 0.01, 0.1, and one copy of the viral genome.

biopsy. The ROSA26 promoter is ubiquitously active (17), and LacZ expression was also found in many organs (brain, liver, kidney, and testis) of the offspring (Fig. 4*C*). Normal offspring were produced from both males and females, and the parental genotype was transmitted in a Mendelian manner to subsequent generations after natural mating (Fig. 4*F*).

To determine whether the adenovirus DNA integrated into the genome of the offspring, DNA was collected from placentas or tails of the offspring and analyzed by PCR and Southern blotting, using the probe from adenovirus genome. Viral DNA was not detected in any of the 21 offspring using either method, indicating that the adenovirus vector did not integrate into the germ line (Fig. 5).

Discussion

Previous attempts failed to demonstrate the infection of spermatogenic cells by adenovirus vectors. In our recent study we found that introduction of adenovirus into the seminiferous tubules resulted in transduction of Sertoli cells, but there was no evidence of infection in germ cells, which are more abundant in the testes (13). This occurred even though germ cells express adenovirus receptors (30, 31). Likewise, other *in vivo* and *in vitro* studies also reported that only somatic cells, but not germ cells, are infected (9–16). Nevertheless, the current study now demonstrates that spermatogonial stem cells are susceptible to adenovirus infection.

Several factors led to successful adenovirus infection in spermatogonial stem cells. First, we used a sensitive reporter mouse strain for virus infection (17). Previous studies have relied on marker gene expression from virus vector or DNA analysis. Although these methods successfully detected retrovirus infection, we assumed that they might fail to detect transient infections or small amounts of infective viruses. In contrast, deletion of floxed sequences in the host genome is irreversible and does not require the presence of adenovirus at the time of detection. Second, we used a chicken β-actin and cytomegalovirus enhancer promoter that has been used to express exogenous genes in spermatogenic cells (32, 33). It is known that germ-line cells suppress viral promoters, and the use of these in previous studies may be one reason that adenovirus infection was not detected (34). Third, we used a higher concentration of spermatogonial stem cells. Previous studies used adult testes, in which the concentration of stem cells is significantly low (0.2–0.3%) (1, 2). In contrast, we used a single-cell suspension prepared from immature testis or GS cells, which lack differentiated germ cells and are more enriched for spermatogonial stem cells, leading to highly efficient transduction.

Table 1. *In vitro* microinsemination with spermatogenic cells recovered from recipient W mice

Type of cells injected	No. of embryos transferred*	No. of embryos implanted (%)	No. of pups (%)
Round spermatid	19	10 (53)	0 (0)
Elongated spermatid	6	4 (67)	0 (0)
Testicular sperm	50	27 (54)	19 (38)
Epididymal sperm	10	5 (50)	2 (20)
Total	85	46 (54)	21 (25)

Data are combined results from three recipient animals.
*Embryos were cultured for 24 h and transferred at the two-cell stage.

An important aspect of adenovirus-mediated gene delivery is its high transduction efficiency. Because it has been considered that spermatogenic cells are resistant to adenovirus infection, our current results were unexpected. In the present study most of the GS cell colonies showed EGFP expression after overnight incubation with AxCANEGFP; in the most successful case, 79% of the cells underwent Cre-mediated deletion. This far exceeds the efficiency of other methods, in which 2–30% of spermatogonial stem cells can be transduced (4, 5, 19). In contrast, previous studies have reported that adenovirus cannot infect spermatogenic cells even when they are exposed to high concentrations of adenovirus (15, 16). This discrepancy suggests that spermatogonial stem cells may differ from more differentiated spermatogenic cells in their susceptibility to adenovirus infection. Given that adenovirus cannot infect mature sperm even when they are exposed to high concentrations of adenovirus (16), it is possible that male germ cells acquire resistance to viral infection as they mature. On the other hand, a potential drawback of the present approach is its toxicity. In the present study adenovirus also influenced the growth rate at high concentrations of adenovirus, which could interfere with the genetic manipulation of spermatogonial stem cells. Nonetheless, the infected cells were still able to differentiate normally after spermatogonial transplantation, indicating that the virus infection did not compromise stem cell function.

Our results have important implications for human gene therapy. Although no evidence of stable adenovirus integration was found in the present study, several lines of evidence have shown that rare integration can still occur after adenovirus infection (at a frequency of 10^{-1} to 10^{-5} per cell) in somatic cells and preimplantation embryos (35, 36), which raises the possibility that stable viral integration may also occur in germ-line cells. Given our results, it may be necessary to reevaluate the frequency of accidental virus insertion using experimental animals. In particular, the possibility of germ-line integration likely increases when the technique is applied to treat male infertility. We previously have rescued infertile male animals with a gene therapy approach, in which a germ cell growth factor is delivered into defective Sertoli cells with an adenovirus vector (13). The injected animals reinitiated spermatogenesis and sired offspring that did not show viral integration. This is currently the only method to rescue infertility because of Sertoli cell defects, and clinical application of this technique may rescue patients with severe hypospermatogenesis or few germ cells. Although the low frequency of stable integration and preferential infection of Sertoli cells *in vivo* suggest that adenovirus vectors may provide a relatively safe approach for treating Sertoli cell-based infertility, our current results indicate that caution is necessary when extrapolating this technique to clinical cases. Further studies are necessary to test whether such stable infection occurs in the male germ line.

One direct application of our results in basic research is using this *in vitro* infection system for analyzing gene functions in spermatogenesis. Although several Cre transgenic lines are available for studying spermatogenesis, very few are available for analysis of the spermatogonia stage, and it is often difficult to study gene functions in spermatogonial stem cells because they are identified only by their function to self-renew. In this sense, adenovirus-mediated Cre expression in spermatogonial stem cells may be useful. By transplanting transgenically marked cells, it is possible to visualize the pattern and kinetics of the repopulation process from single stem cells (27), which can reveal abnormal functions that are not evident in physiological conditions. The Cre infection may also be applied for selectable marker removal after gene targeting in GS cells (37) or for more sophisticated genetic modifications. For example, a transposon/transposase construct may be used as a cargo for adenovirus, and the ensuing transient expression of the cargo should generate an active transposase, which then inserts the transposon cargo permanently in the germ line. Thus, the unique mode of gene delivery by adenovirus vectors complements previously established genetic manipulation methods that achieve stable germline integration and will provide new opportunities for studies on male germ cell biology.

Materials and Methods

Recombinant Adenovirus. The replication-defective adenovirus vectors AxCANLacZ and AxCANCre were obtained from RIKEN. AxCANEGFP was a generous gift from I. Saito (University of Tokyo, Tokyo, Japan). These vectors used the cytomegalovirus enhancer promoter, which can be expressed in spermatogonial stem cells (32, 33). The viruses were purified from 293 cells by using CsCl centrifugation. The titer of the virus was 2×10^8 pfu/ml, which was diluted before use.

Animals and Cell Culture. For *in vivo* infection, purified adenovirus vector (1×10^6 pfu/ml) was microinjected into the seminiferous tubules of ICR mice that were 5–10 days and 5 weeks old (Japan SLC, Shizuoka, Japan). For *in vitro* infection, adenovirus was exposed to primary testis cells that were recovered from 6-day-old ICR mice or GS or mGS cells established from newborn DBA/2 mice (21, 22). All primary testis cells were maintained on mouse embryonic fibroblasts in Stempro34 medium (Invitrogen, Carlsbad, CA), as described (21). mGS cells were maintained in DMEM/15% FCS with leukemia inhibitory factor on gelatin-coated plates, and GS cells were cultured on laminin in Stempro34 medium, as described (38). In some experiments, we used testis cells from a R26R mouse that was kept in B6 background (The Jackson Laboratory, Bar Harbor, ME) (17). In infection of primary testis cells, 1×10^6 cells were plated in a 6-well plate (9.5 cm^2), whereas 3×10^5 cells were plated in a 12-well plate (3.8 cm^2) in infection of GS and mGS cells. The cells were incubated overnight with adenovirus at concentrations ranging from 6×10^3 to 6×10^5 pfu/ml. The *in vitro* deletion efficiency was estimated by using homozygous R26R mice, whereas heterozygous R26R mice were used to examine germ-line integration in the offspring. The cultured cells were transplanted into WBB6F1-W/W^v (W) mice that do not have endogenous spermatogenesis because of mutations in the *c-kit* gene (39).

Surgical Procedure. Microinjection into the seminiferous tubules was performed via the efferent duct. Each injection filled 75–85% of the seminiferous tubules in each testis (40). Approximately 10 μl was introduced into the ICR testes, whereas only 2 or 4 μl could be introduced into W mice that were 5–10 days or 4 weeks old, respectively. When recipient mice were not histocompatible with the transplanted cells, they were treated with anti-CD4 antibody to induce tolerance to the donor cells (41). The Institutional Animal Care and Use Committee of Kyoto University approved all of the animal experimentation protocols.

Analysis of Transgene Expression. In experiments using AxCANLacZ, the tissues were fixed in 4% paraformaldehyde for 2 h, and X-Gal staining was used to detect LacZ expression (27). The same procedure was used to detect LacZ expression in R26R mice after Cre recombination. In experiments using AxCANEGFP, cells were analyzed by a microscope equipped with UV fluorescence (21). For flow cytometry, the cultured cells were dissociated by trypsin, and single-cell suspensions were incubated with rat anti-mouse EpCAM antibody (G8.8; BD Biosciences, Franklin Lakes, NJ), which was detected by allophycocyanin-conjugated anti-rat IgG antibody (BD Biosciences), as described previously (42). The cells were analyzed by a FACSCalibur system (BD Biosciences).

Histological Analysis. The testes were fixed with 10% neutral-buffered formalin and processed for paraffin sectioning. Two histological sections were made from each recipient testis with an interval of 12 μm between sections. All sections were stained with hematoxylin and eosin.

DNA Analysis. Genomic DNA was isolated from cultured cells or tissue samples by phenol/chloroform extraction, followed by ethanol precipitation. The deletion of the floxed allele was estimated by PCR using the 5′-TTTCTGGGAGTTCTCTGCTGC-3′ and 5′-TCACGACGTTGTAAAACGACG-3′ primers.

To estimate the efficiency of Cre-mediated deletion, a 270-bp fragment in the ROSA26 promoter region was amplified by PCR using the 5′-CCTAAAGAAGAGGCTGTGCTTTGG-3′ and 5′-CGTCCGGTGGAGACTTTTC-3′ primers, which were used as a hybridization probe. Twenty micrograms of DNA was digested with restriction enzymes and separated on a 1.0% agarose gel. DNA transfer and hybridization were performed as described previously (33). To detect the adenovirus genome in offspring, a 15,043-bp ScaI-EcoRI fragment of pAxCAiLacZit cosmid vector was used as a hybridization probe (Nippon Gene, Toyama, Japan). The intensity of bands was quantified by NIH Image 1.63. To detect virus integration, a 310-bp region of adenovirus type 5 was amplified by PCR using specific primers, as described previously (13).

Microinsemination. The seminiferous tubules of recipient mice were mechanically dissected, and spermatogenic cells were collected. Microinsemination was performed by intracytoplasmic injection, as described previously (28). Embryos that reached the two-cell stage after 24 h in culture were transferred to the oviducts of day-1 pseudopregnant ICR female mice. Fetuses that were retrieved on day 19.5 were raised by ICR foster mothers.

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Leukemia Inhibitory Factor Enhances Formation of Germ Cell Colonies in Neonatal Mouse Testis Culture[1]

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ABSTRACT

Spermatogonial stem cells continuously divide in the testis to support spermatogenesis throughout the life of adult male animals. Although very few spermatogonial stem cells are present in vivo, we recently succeeded in expanding these cells in vitro. Germ cells from postnatal testes were able to proliferate in the presence of several types of cytokines, and they formed uniquely shaped colonies of spermatogonia (germline stem or GS cells). These cells reinitiated normal spermatogenesis when transplanted into seminiferous tubules. However, much remains unknown about the contributions of cytokines to successful stem cell culture. In the present study, we examined the role of leukemia inhibitory factor (LIF) in GS cell culture. We found that the addition of LIF to newborn testis cell culture enhances the formation of germ cell colonies. Ciliary neurotrophic factor, but not oncostatin M, had the same effect, although they both bind to the IL-6ST (gp130) receptor. On the other hand, GS cells could be established from pup or adult testes in the absence of LIF. No phenotypic or functional difference was found between GS cells established from different stages, and normal offspring were born from pup-derived GS cells that had been maintained in the absence of LIF, indicating that LIF per se is not involved in the self-renewal of GS cells. These results demonstrate that LIF is useful in the initiation of GS cell culture and suggest that LIF or a related cytokine is involved in the maturation of gonocytes into spermatogonia.

developmental biology, gametogenesis, Sertoli cells, spermatogenesis, testis

INTRODUCTION

Male germline development is a long and complex process [1]. In mice, precursors of fetal germ cells originate from the epiblast and can be detected at the base of the allantois by alkaline phosphatase activity at about 7.5 days postcoitum (dpc) [2]. In males, the fetal germ cells, called primordial germ cells (PGCs), increase in number and migrate to the gonad by 12.5 dpc. When PGCs enter the gonad, they become enclosed in seminiferous cords and are then called gonocytes. These gonocytes proliferate for a couple of days and then become mitotically quiescent by about 16.5 dpc. At 1–3 days postpartum (pp), they enter the cell cycle. Although they are uniformly round in the fetal stage, they form pseudopods and begin to migrate toward the basement membrane. By Days 3–7 pp, type A spermatogonia appear in the seminiferous tubules, and a small number of these spermatogonia exhibit the unique proliferative potential both to self-renew and differentiate. In differentiation, the spermatogonia produce spermatocytes, and the latter cells go through meiosis to produce spermatozoa, thereby supporting spermatogenesis throughout adult life [3]. Although the morphological aspects of the dynamic development of the male germ cell system have been well described, much remains unknown about the developmental mechanisms involved and their regulation.

Leukemia inhibitory factor (LIF) plays an important role in the regulation of stem cells. LIF binds to a heterodimeric receptor (LIF receptor) that includes the LIF-specific binding subunit and the transmembrane signal-transducing subunit IL-6ST. Binding of LIF to this receptor complex results in the phosphorylation of several tyrosine residues in IL-6ST by the activated Janus kinase (JAK) tyrosine kinases, which then activates signal transducer and activator of transcription (STAT) 3 transcription factor [4, 5]. This signaling cascade has been shown to be involved in the maintenance or self-renewal of stem cells in several tissues. For example, a decrease in the number of neural stem cells was found in LIF receptor-deficient mice [6], and the introduction of dominant negative STAT3 molecules was found to negatively influence hematopoietic stem cell potential [7]. In embryonic stem (ES) cells, LIF acts to activate STAT3 and maintain the undifferentiated state and allows their efficient propagation in vitro [8, 9]. LIF also promotes the survival or proliferation of PGCs, from which the ES-like pluripotent cells, known as embryonic germ (EG) cells, have been derived from in vitro experiments [10, 11]. In contrast, although LIF receptors are also found on gonocytes and spermatogonia at all postnatal ages [12, 13], the involvement of LIF in postnatal germ cell development has been unclear.

We recently developed a culture system for spermatogonial stem cells. As we originally reported, gonocytes from neonatal mouse testes proliferated to form uniquely shaped colonies of germ cells when cultured in the presence of glial cell line-derived neurotrophic factor (GDNF) and LIF [14]. Similarly, when spermatogonia from pup or adult testes were directly seeded on LIF-secreting feeder cells or in LIF-containing medium with the addition of GDNF, the cultured cells continued to proliferate logarithmically [15, 16]. Even after

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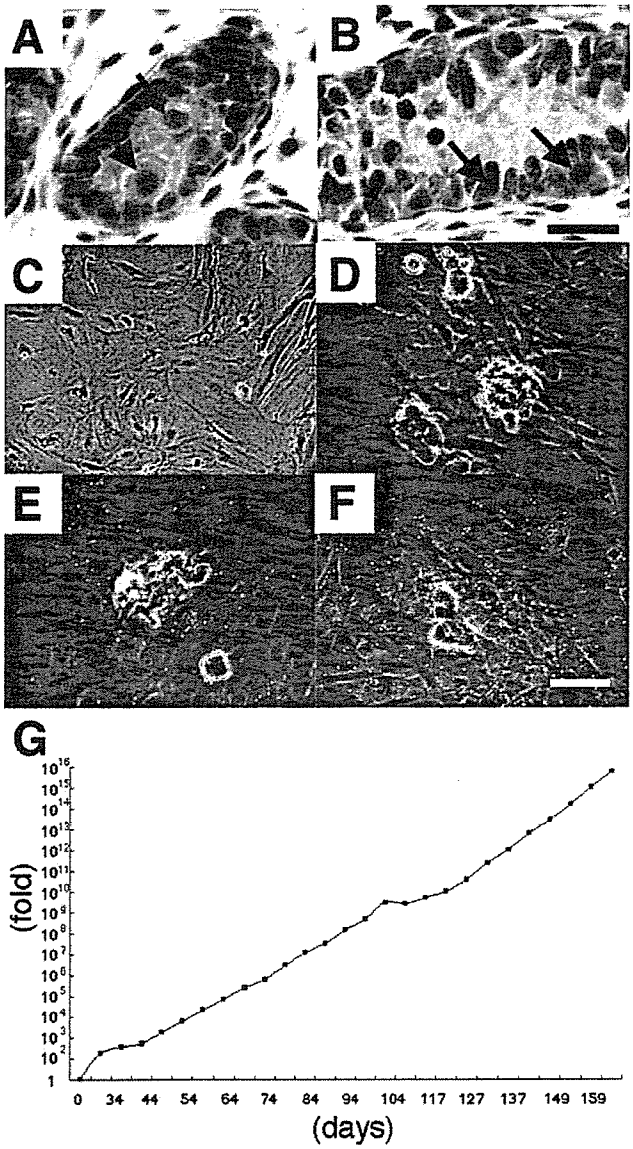

FIG. 1. Initiation of germ cell culture from neonatal and pup testes. **A**, **B**) Histological appearance of mouse testes on Day 0 (**A**) and Day 3 (**B**). Note the absence of proliferation on Day 0 pp. Arrows indicate germ cells. **C**, **D**) Neonatal testis cells cultured in the absence (**C**) or presence (**D**) of LIF for 9 days. Note the growth of germ cells in the presence of LIF (**D**). **E**, **F**) Pup testis cells cultured in the absence (**E**) or presence (**F**) of LIF for 5 days. **G**) Proliferation of GS cells cultured from pup testes in the absence of LIF. Small gold objects are magnetic beads that were used to isolate spermatogonial stem cells. Bar = 20 μm (**A**, **B**) and 50 μm (**C**–**F**).

2 yr of in vitro culture, the cultured cells initiated long-term spermatogenesis and produced normal offspring when transplanted into the seminiferous tubules of infertile recipient mice [17]. On the basis of these properties, we designated these cells germline stem or GS cells [14]. Although GS cells were originally established from neonatal gonocytes, they express spermatogonia markers and show functional characteristics of spermatogonia. The observation that, under the same culture conditions, similar cells could be established from spermatogonia of pup and adult testes indicates that gonocytes can differentiate into spermatogonia during culture [16].

Although GDNF has been shown to be a self-renewal factor for spermatogonial stem cells, the role of LIF in GS cell culture is not well understood. In the present investigation, we cultured germ cells collected at different stages of testicular development, and the stage-specific effects of LIF were analyzed by phenotypic and functional analysis of spermatogonial stem cells.

MATERIALS AND METHODS

Cell Culture

For the initiation of testis cell culture, Institute for Cancer Research (ICR) mice were purchased from Japan SLC (Shizuoka, Japan). In experiments to visualize spermatogonia cells, we used *Neurog3* (*ngn3*)/enhanced green fluorescent protein (*EGFP*) transgenic mice from a DBA/2 background [18]. In long-term culture experiments, we used the transgenic mouse line C57BL6/Tg14(act-EGFP-OsbY01) bred into the DBA/2 background (designated Green; provided by Dr. M. Okabe [Osaka University]) [19].

Testis cells were collected at various time points by two-step enzymatic digestion with collagenase and trypsin [20], and cell culture was performed according to the previously published protocol [14]. In some experiments, anti-CD9 antibody was used to enrich spermatogonial stem cells from pup and adult testes [21]. The growth factors used were mouse epidermal growth factor (20 ng/ml; BD Biosciences, Franklin Lakes, NJ), human basic fibroblast growth factor (10 ng/ml; BD Biosciences), and recombinant rat GDNF (10 ng/ml; R&D Systems, Minneapolis, MN). In some cases, murine LIF (10^3 U/ml; Invitrogen, Carlsbad, CA), rat ciliary neurotrophic factor (CNTF; 100 ng/ml), or mouse oncostatin M (10 ng/ml [OSM]; both from R&D Systems) was added to the medium. Cells were cultured in the presence of 1% fetal bovine serum (JRH Biosciences, Lenexa, KS). After the second or third passage, cells were maintained on mouse embryonic fibroblasts.

Germ cell colonies were quantified by analyzing individual wells of the culture plate. A cluster of germ cells was defined as a colony when it contained >16 cells. In the fetal and neonatal cell cultures, cells were plated in a 12-well plate (2×10^5 cells/3.8 cm^2), and the cultures were assayed at Day 10 after the initiation of culture. In the pup and adult cell cultures, CD9-expressing cells were plated in a six-well plate (2×10^5 cells/9.5 cm^2), and the cultures were assayed at Days 6 and 9, respectively, because of the extensive proliferation of testicular somatic cells.

Transplantation Procedure

For testicular injection, cultured cells were dissociated by trypsin and suspended in Dulbecco modified Eagle medium with 10% fetal calf serum, supplemented as described [20]. Approximately 3 μl of single-cell suspension containing 1.5×10^4 cells was introduced into the seminiferous tubules of 4- to 10-wk-old WBB6F1-W/W^v (W) recipient mice, which are congenitally infertile because of mutations in the *c-kit* tyrosine kinase receptor [22]. Microinjection was performed by the efferent duct injection method [20], which filled 75%–85% of the tubules in each recipient testis. Because the recipient mice were not histocompatible with the transplanted cells, they were treated with anti-CD4 antibody to induce tolerance to the donor cells [23]. The Institutional Animal Care and Use Committee of Kyoto University approved all of the animal experimentation protocols.

RT-PCR Analysis

Total RNA was isolated from the cultured cells or testis cells with Trizol reagent (Invitrogen). First-strand cDNA was synthesized with Superscript II (RNase H^- reverse transcriptase, Invitrogen). PCR was carried out with specific primers (5′-TCAGGACTTCAAGGATAGTGAGG-3′ and 5′-AAGGCATGAGAGAGAGCACC-3′ for *Osm*, 5′-TCCGTTGTGAGATGGAAGG-3′ and 5′-GAATCATCCGGTGTACTGCC-3′ for *Cntf*, and 5′-CCTCTTCCCATCACCCCTGTAAAT-3′ and 5′-ACTTGGTCTTCTCTGTCCCGTTGC-3′ for *Lif*). Other primers were as previously described [17].

Analysis of Testes

Donor cell colonization was analyzed by UV fluorescence microscopy [14]. Because the host testis does not have endogenous fluorescence, this method allows the specific identification of transplanted cells. A cluster of germ cells was defined as a colony when it occupied more than 50% of the basal surface of the tubule and was at least 0.1 mm in length. The efficiency of colonization was evaluated by counting the total number of colonies under a stereomicroscope equipped with UV light. For histological analysis, the recipient testes were also

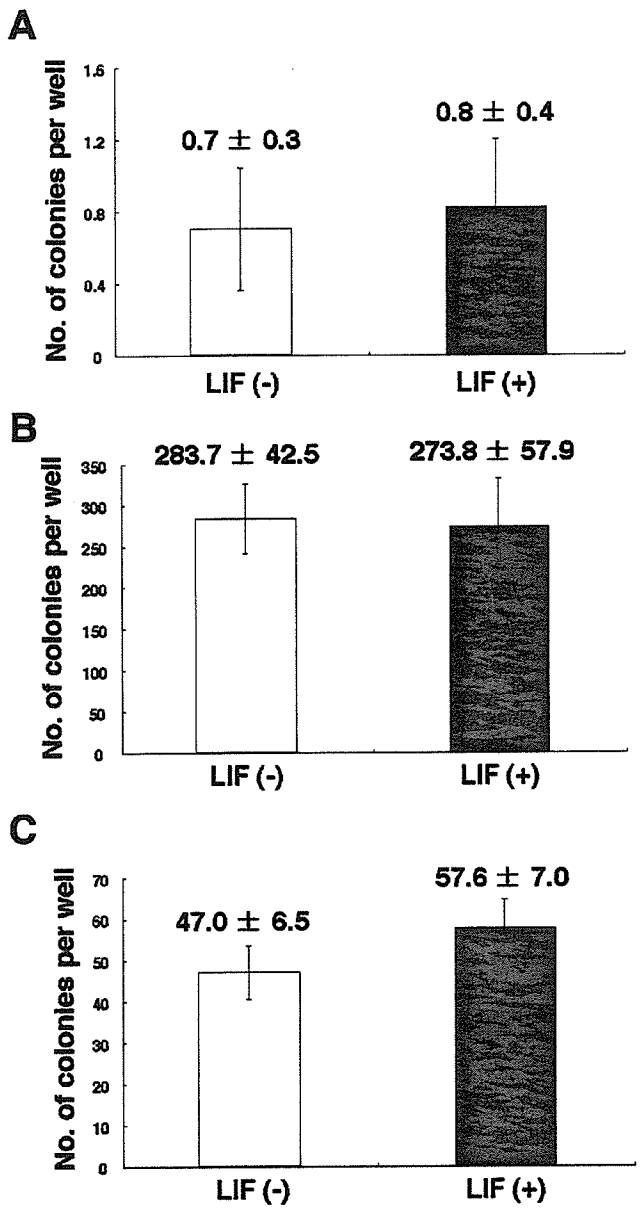

FIG. 2. The effect of LIF on the formation of germ cell colonies from (A) fetal (14.5 dpc), (B) pup, and (C) adult mouse testes. Means ± SEM for at least six samples in two to three experiments are shown. No statistical difference could be found by *t*-test analysis ($P < 0.05$).

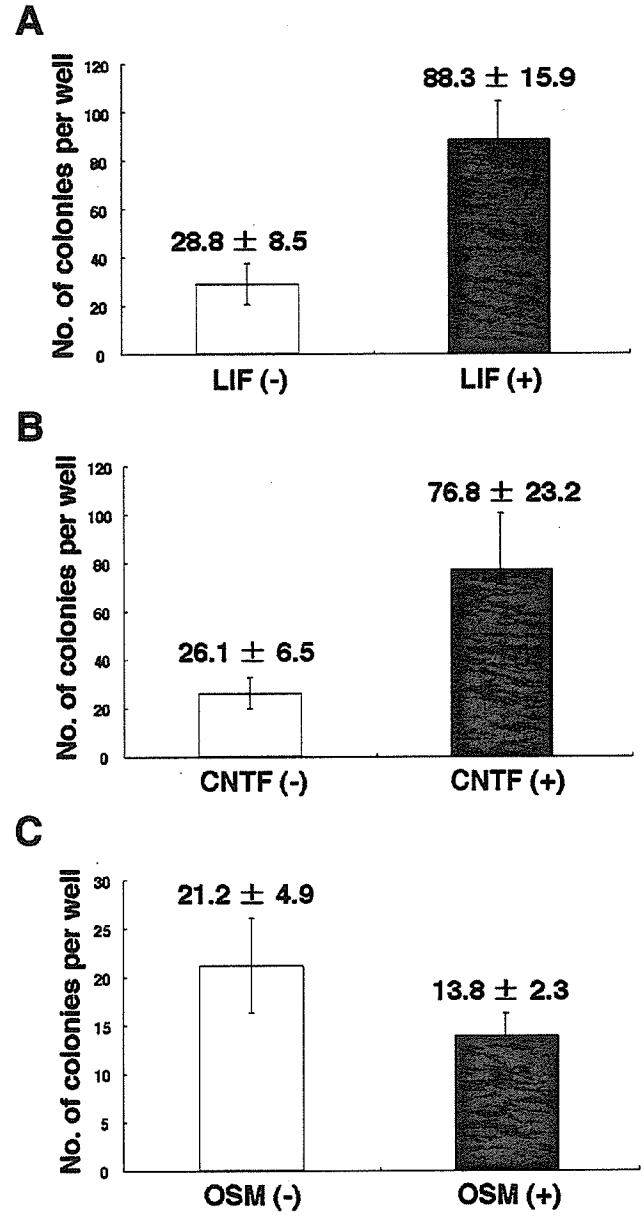

FIG. 3. The effect of LIF (A), CNTF (B), and OSM (C) on the formation of germ cell colonies from a neonatal mouse testis. Means ± SEM for at least 11 samples in three experiments are shown. All values were statistically significant by *t*-test analysis ($P < 0.05$).

fixed in 10% neutral-buffered formalin and processed for paraffin sectioning. All sections were stained with hematoxylin and eosin. Statistical analysis was performed by the Student *t*-test.

Flow Cytometry

The primary antibodies used were as follows: rat anti-EpCAM (G8.8) and mouse anti-SSEA-1 (MC-480; Developmental Studies Hybridoma Bank, University of Iowa, Ames), rat anti-human INTA6 (CD49f) (GoH3), biotinylated hamster anti-rat INTGB1 (CD29) (Ha2/5), biotinylated rat anti-CD9 (KMC8), allophycocyanin (APC)-conjugated rat anti-mouse c-kit (CD117) (2B8) (BD Biosciences), and rat anti-TDA (EE2) (provided by Dr. Y. Nishimune, Osaka University). APC-conjugated goat anti-rat immunoglobulin G (IgG; Cedarlane Laboratories, Ontario, TN, Canada), APC-conjugated streptavidin (BD Biosciences), or Alexa Fluor 633-conjugated goat anti-mouse immunoglobulin M (IgM; Molecular Probes, Eugene, OR) was used as the secondary antibody. Cells were stained as previously described [24] and analyzed with a FACS-Calibur system (BD Biosciences), with 10 000 events acquired.

Western Blot Analysis

Samples were separated by SDS-PAGE, transferred to polyvinylidene fluoride membranes (Hybond-P; Amersham Pharmacia Biosciences, Buckinghamshire, U.K.), and probed with rabbit polyclonal anti-STAT3-P (tyrosine 705) antibody (Cell Signaling, Danvers, MA). Peroxidase-conjugated anti-rabbit IgG antibody was used as the secondary antibody (Cell Signaling).

Microinsemination

The seminiferous tubules of recipient testes were dissected under UV illumination when the recipient mice were 7 mo old. The EGFP-expressing

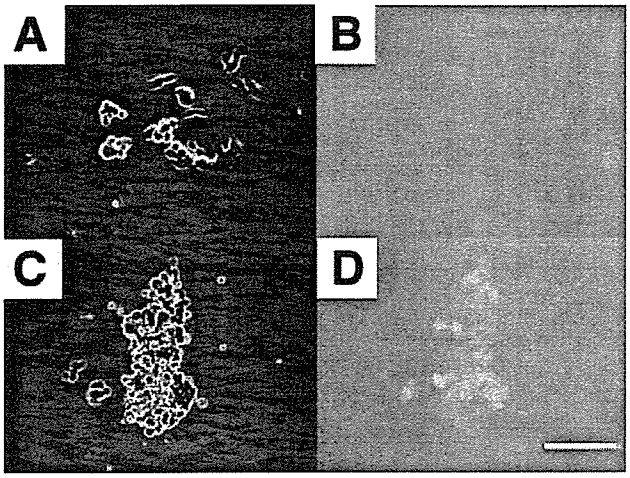

FIG. 4. Derivation of GS cell colonies from neonatal ngn3-EGFP transgenic mouse testes. **A**, **B**) Neonatal testis cells cultured in the absence of LIF for 23 days. No fluorescence was detected under UV light (**B**). **C**, **D**) Neonatal testis cells cultured in the presence of LIF for 23 days. EGFP-positive ngn3-expressing spermatogonia could be detected under UV light (**D**). Bar = 200 μm.

seminiferous tubules were recovered, and the germ cells were collected mechanically from the tubules with fine forceps. Microinsemination was performed as described previously with round spermatids [25]. Round spermatids were used in the present experiments because they were more readily recovered from the recipient testes. Embryos that reached the two-cell stage after 24 h in culture were transferred to the oviducts of Day 1 pseudo-pregnant ICR female mice. Fetuses that were retrieved on Day 19.5 were raised by ICR foster mothers.

RESULTS

LIF Promotes Formation of GS Cell Colonies from Neonatal Testes

To examine the effect of LIF on gonocytes, we cultured fetal or newborn testis cells in the presence or absence of LIF. The only germ cells present at this stage are gonocytes, which are separated from the basement membrane. Although they are mitotically quiescent and differ morphologically from spermatogonia, they have spermatogonial stem cell potential and the ability to colonize empty seminiferous tubules after germ cell transplantation [26, 27]. In ICR strains, gonocytes are found in the center of the seminiferous tubules at the time of birth (Fig. 1A), and they migrate to the basement membrane by 3 days after birth (Fig. 1B). Genital ridges or testes were collected from 14.5-, 16.5-, and 18.5-dpc embryos, and testes were collected from 0- to 2-day-old newborn animals. The tissue fragments were dissociated by enzymatic digestion to obtain single cells.

To enrich germ cells, the dissociated cells were plated at a density of 2×10^5 cells/3.8 cm^2 on gelatin-coated plates. After overnight incubation, some of the plated cells had adhered to the culture dish. The floating cells were recovered by pipetting the next day and transferred to a new tissue culture plate at 1:1 dilution. In 2–3 days, floating gonocytes began to attach to the somatic cells, and the germ cells started to form colonies. The formation of germ cell colonies from newborn testes was significantly reduced in the absence of LIF (Fig. 1, C and D). Proliferating cells were observed in cultures from genital ridges or testes from 18.5-dpc embryos and newborn mice in 7–10 days, but very few proliferative cells were observed in cultures from the genital ridges of 14.5- or 16.5-dpc embryos, even in the presence of LIF.

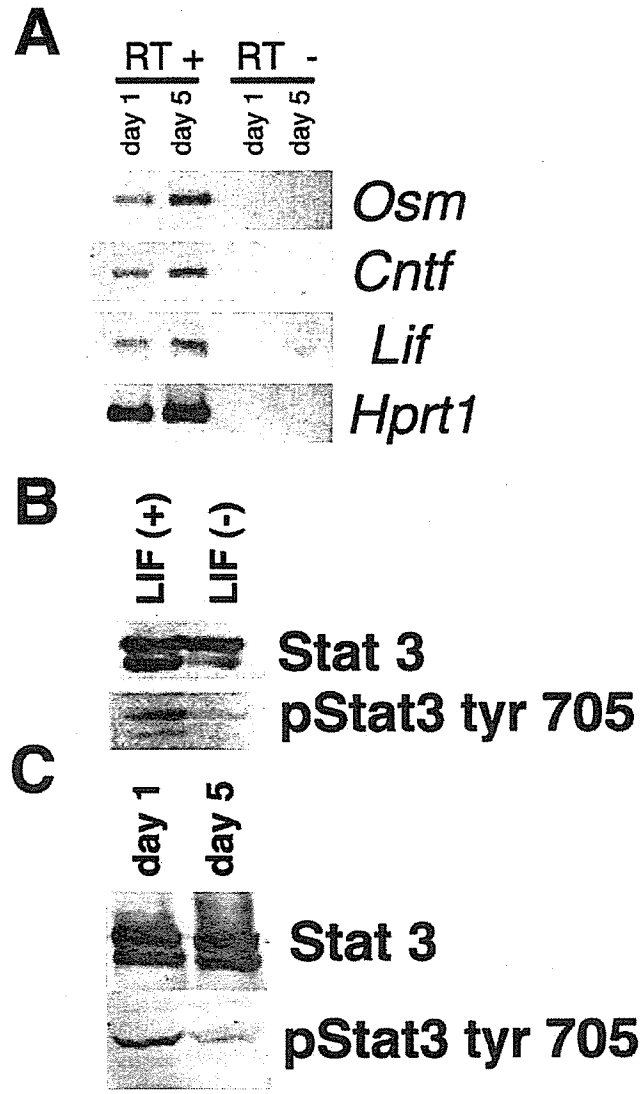

FIG. 5. Expression of LIF and other members of the IL-6 family in the neonatal and pup testes. **A**) RT-PCR analysis of neonatal and pup testes. **B**) Western blot analysis of GS cells cultured on a laminin-coated dish. Phosphorylation of STAT3 was detected in the presence of LIF. **C**) Western blot analysis of neonatal and pup testes. Phosphorylation of STAT3 was detected in the testes of 1-day-old neonates. The signal was weaker in the testes of 5-day-old pups.

To quantify the effect of LIF on germ cell colony formation, we counted the number of germ cell colonies containing >16 cells. Although LIF did not have a significant effect on colony formation in fetal germ cell culture (Fig. 2A), the addition of LIF increased the number of germ cell clusters by 3.1-fold when the cultures were analyzed 10 days after culture initiation (Fig. 3A). To examine whether the germ cells differentiated into spermatogonia, we used ngn3-EGFP transgenic mice that expressed EGFP in A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia [18]. In the presence of LIF, cells in the colonies displayed EGFP fluorescence, indicating that the gonocytes had already differentiated to spermatogonia by the time of analysis. On the other hand, few EGFP-positive cells were present in the culture when LIF was absent (Fig. 4).

In contrast, LIF did not have a significant effect on colony formation from spermatogonia. Testis cells from 5-day-old

TABLE 1. Spermatogonial stem cell expansion in LIF-free culture.*

Experiment	Days to transplant[a] (passage)	Colonies/testis	Colonies/10^5 GS cells	Increase in cell number[b] (fold)	Increase in stem cell number[b] (fold)
1	84 (16)	11.2 ± 2.4	74.7 ± 16.1		
2	104 (20)	1.2 ± 0.3	7.8 ± 2.1	82.0	8.6
3	149 (28)	6.0 ± 0.8	40.0 ± 5.5	4.8×10^6	2.6×10^6

* Values are mean ± SEM. In each experiment, 1.5×10^4 cells were microinjected into the seminiferous tubules of infertile recipient testis. Results from four to six recipient testes for each transplantation.
[a] The number of days from initiation of culture to transplantation.
[b] The increase in the total cell or stem cell number from the initial transplantation (experiment 1).

pups and 4-wk-old adults were dissociated by enzymatic digestion, and anti-CD9 antibody was used to select CD9-expressing spermatogonia, which contain spermatogonial stem cells [21]. When the selected cells were directly plated on a tissue culture plate, spermatogonia from both the pup and adult testes proliferated and formed colonies in the absence of LIF (Fig. 1, E and F). We did not observe a significant difference in the morphology or frequency of colony formation between LIF-treated and -untreated cultures (Fig. 2, B and C), indicating that LIF does not influence the initiation of culture with spermatogonia. In agreement with these results, the effect of LIF on GS cell colony formation was limited to the initiation phase of neonatal testis cell culture. The GS cell colonies derived without LIF from neonatal testes could be passaged, and the GS cells derived with LIF continued to proliferate when LIF was removed at 2 or 3 wks after the initiation of culture (data not shown).

Because LIF is a member of the interleukin 6 (IL-6) superfamily, we examined whether other IL-6 family members could enhance germ cell colony formation from gonocytes. We chose to test CNTF and OSM, both of which belong to the IL-6 superfamily and can bind to the IL-6ST receptor [4, 28]. The CNTF and OSM are expressed in neonatal and pup testes (Fig. 5A). Interestingly, the effect of LIF was mimicked by CNTF, which increased the number of colonies 2.9-fold, whereas the addition of OSM did not have a significant effect on colony formation, decreasing to only 0.7-fold in 10 days (Fig. 3, B and C). Although the morphology or proliferation patterns of germ cell colonies in these cultures were similar to those that developed in the presence of LIF, in the presence of OSM, germ cell colonies disappeared within 2–3 wk because of the prolific growth of somatic cells. Phosphorylated STAT3, which transmits signals from the LIF receptor [4, 5], was found not only in the GS cells but also in the neonatal and pup testes by Western blotting (Fig. 5, B and C), suggesting that ligand binding and activation of downstream molecules also occurs in vivo.

Phenotypic and Functional Analysis of Cultured Cells

We next examined the phenotypic characteristics of GS cells that were established from 5-day-old Green mouse pup testes in the absence of LIF. When these cells were examined for the expression of cell surface markers by flow cytometry (Fig. 6A), they were found to express several spermatogonial markers strongly, including INTGB1 and INTA6, CD9, EpCAM, and EE2 [21, 24, 29, 30]. KIT, a marker for differentiated spermatogonia [31], was expressed in varying amounts. However, the cultured cells never expressed SSEA-1 or Forssman antigen, each of which is a marker of PGCs [32, 33]. Results from RT-PCR also confirmed the spermatogonia phenotype of the pup testis-derived GS cells (Fig. 6B). The cells expressed spermatogonial markers, such as *Pou5f1* (Oct-4), *Zfp42* (Rex-1), *Ret* (c-ret), *Neurog3* (ngn3), *Ddx4* (Mvh), and *Zbtb 16* (PLZF) [18, 34–39], but did not express Nanog, a marker of pluripotent cells [40, 41]. Overall, these features are similar to those of GS cells cultured in the presence of LIF [14]. In addition, neither the colony morphology nor the rate of proliferation was influenced by the addition of LIF, OSM, or CNTF, and the cells were passaged at a ratio of 1:3 to 1:5 every 5–6 days. No effect was observed for LIF, even when the cells were cultured in feeder-free or suspension culture conditions. Similar results were found for GS cells established from adult testes in the absence of LIF (data not shown).

To address whether the cultured cells contained spermatogonial stem cells, spermatogonial transplantation was used to assess the cells for their ability to colonize and reinitiate spermatogenesis in empty seminiferous tubules [42]. Cells were collected at three different time points after the initiation of culture and microinjected into the seminiferous tubules of infertile W recipient mice. The recipient testes were analyzed 2 mo after transplantation by detection of EGFP-positive colonies under UV light (Fig. 7A). While the total cell number increased 4.8×10^6-fold during the 65-day period after initial transplantation, the number of spermatogonial stem cells, as assessed by the functional transplantation assay, increased 2.6×10^6-fold. Because the number of stem cells in the pup testis is $5/10^5$ cells [43], this result also indicates that stem cells expanded at least approximately 1.4×10^{13}-fold from the initiation of culture to 149 days, while the total cell number increased by 1.7×10^{12} after stem cell purification and in vitro culture (Table 1 and Fig. 1G). When histological sections of the recipient testes were examined, these colonies contained normal-appearing spermatogenesis (Fig. 7B).

Finally, to determine whether the germ cells that developed from LIF-free GS cells were functionally normal, we used in vitro microinsemination, a technique commonly used to produce offspring from infertile animals and humans [25, 44]. Germ cells were recovered from the EGFP-positive seminiferous tubules by repeated pipetting (Fig. 7, C and D). Oocytes derived from C57BL/6 × DBA/2 F1 females were microinjected with round spermatids collected from two different recipient W mice. A total of 49 eggs were cultured for 24 h, and 30 embryos were transferred into the oviducts of pseudo-pregnant ICR host mothers. Ten embryos were implanted successfully in the uteri, and five offspring were born, four males and one female (Fig. 7E). The average body and placental weights of the offspring were 1.63 and 0.15 g, respectively, both of which are within the reference range. Of the five offspring, four displayed EGFP fluorescence, indicating their donor origin. Three of the offspring grew up to be normal fertile adults.

DISCUSSION

The immature testis is a good source for spermatogonial stem cell culture because it lacks differentiated germ cells and is enriched for spermatogonial stem cells. In particular, in the neonatal testis, germ cells are separated from the basement

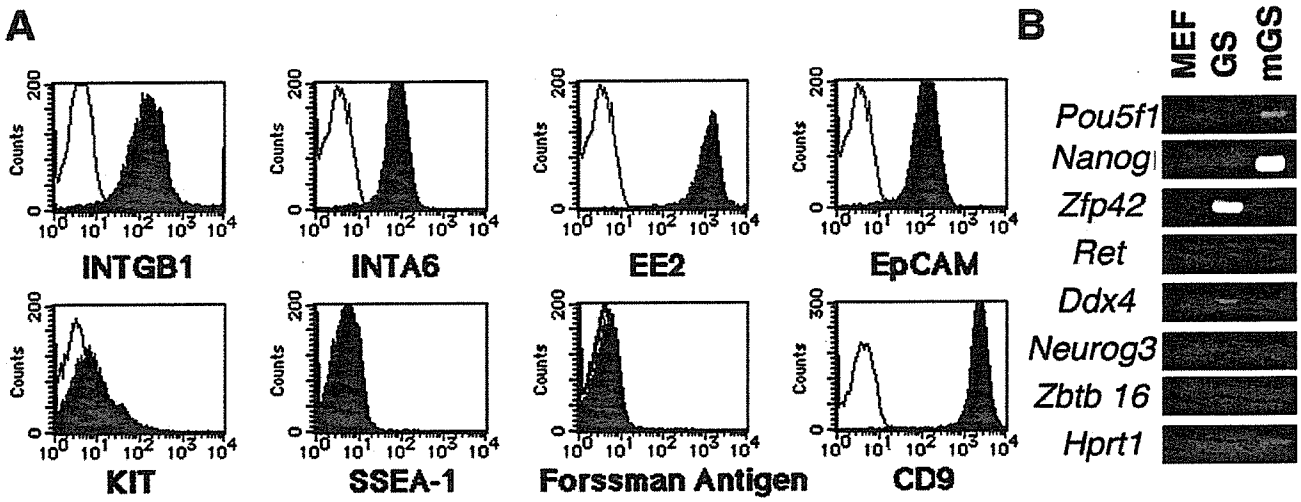

FIG. 6. Phenotypic analysis of GS cells cultured in the absence of LIF. A) Characterization of cell surface antigen by flow cytometry. EE2 and EpCAM are surface antigens that are expressed on spermatogonia. Black line, specific antibody; broken line, unstained control. B) RT-PCR analysis of GS cells. Specific primers were used to amplify cDNA from mouse embryonic fibroblasts (MEFs), GS cells, and multipotent germline stem (mGS) cells.

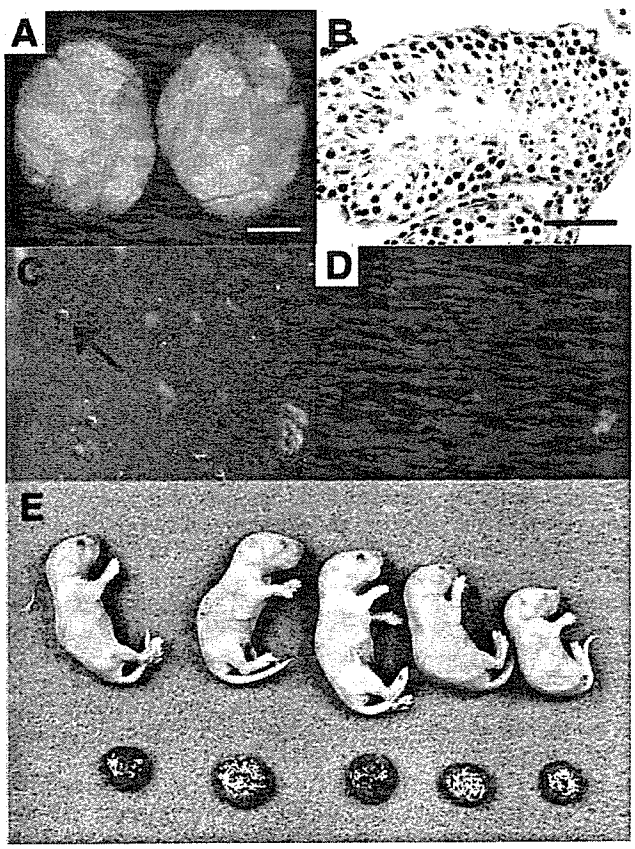

FIG. 7. Production of fertile offspring from GS cells cultured in the absence of LIF. A) Recipient testes transplanted with EGFP-expressing GS cells that were cultured in the absence of LIF. B) Histological analysis of the recipient testis. Normal-appearing spermatogenesis was observed. C, D) Spermatogenic cells released from a segment of seminiferous tubule that was colonized by EGFP-positive donor cells. Nomarski (C) and fluorescent (D) images are shown. The arrow indicates a donor-derived round spermatid that was used in microinsemination. E) Offspring that developed from microinsemination. Bar = 1 mm (A), 50 μm (B).

membrane and are located in the lumen of immature seminiferous tubules. The only germ cells present at this time are early differentiating spermatogonia and spermatogonial stem cells [45]. Hence, germ cells can be efficiently recovered by simple gelatin selection. In contrast, in the mature testis, spermatogonia are attached to the basement membrane, and the relative frequency of spermatogonial stem cells is very low (usually 0.2%–0.3%) [1, 3]. Thus, the recovery of spermatogonial stem cells from the mature testis requires cell sorting or other purification procedures, which often result in a significant loss or reduced stem cell viability [21, 24]. The favorable characteristics of the neonatal mouse testis were exploited in the initial establishment of the GS cell culture system from neonatal testes [14]. In this system, a cocktail of several cytokines was shown to stimulate germ cell proliferation, but the roles of the individual components have not precisely been evaluated.

In the testis, LIF is produced by the peritubular cells of the seminiferous tubules, and its receptor is expressed on germline cells from fetal stages to spermatogonia in mature testes [12, 13, 46]. On the basis of this information, LIF has often been used in the culture of germline cells. In testis cell culture, LIF was originally shown to promote the survival or proliferation of rat gonocytes; it stimulated the proliferation of quiescent gonocytes isolated from Day 1 testes [47]. However, the proliferation stopped within ~1 wk under those conditions, and LIF did not promote the survival of proliferating gonocytes from 3-day-old rat testes. In another study, a similar clonogenic assay in mice did not show any enhancement of gonocyte proliferation by LIF [48]. These conflicting results may be because of from the suboptimal culture conditions used in these studies. In particular, the lack of GDNF, a self-renewing factor for spermatogonial stem cells [36], in previous studies may have adversely influenced the stem cell conditions or viability. In the present study, we examined the role of LIF by a GS cell culture system in which a self-renewal division of spermatogonial stem cells stably occurs in vitro. In contrast to previous methods that do not maintain spermatogonial stem cells, this culture system allows a more precise estimation of the effects of growth factors on germ cells, because the germ cells are viable long term.

The results of the present study indicate that the effect of LIF on testis culture is stage-specific. Although LIF was able to

stimulate the proliferation of gonocytes in 18.5-dpc to neonatal mice, we were unable to derive GS cells from early gonocytes from 14.5- to ~16.5-dpc fetal gonads by the same protocol, suggesting that late gonocytes more readily convert into spermatogonia. Although gonocytes proliferate until about 16.5 dpc, several functional differences between the early and late stages of gonocytes are indicated. For example, while gonocytes from 12.5-dpc embryos failed to colonize seminiferous tubules after spermatogonial transplantation, those from 18.5-dpc embryos produced normal spermatogenesis [26]. In addition, male germ cells acquire androgenetic imprint patterns during this period [49]. Much information remains unknown about how these functional changes occur in gonocytes, and our results indicate that LIF is apparently insufficient to reconstitute the changes that occur during development.

On the other hand, we found that GS cells can be established and expanded from pup and adult testes in the absence of LIF and, furthermore, that the established cells can be maintained long term in the absence of LIF and contribute to the germline. These features contrast with those of the ES cell system, in which the removal of LIF results in the differentiation and loss of germline potential [8, 9]. Although both types of stem cells can contribute to the germline lineage, ES and GS cells apparently differ in their differentiation potentials and regulatory mechanisms. Our results suggest that the effectiveness of LIF is limited to perinatal gonocytes and that LIF signaling per se does not promote proliferation or maintenance of the undifferentiated state of GS cells once the cells are established. Consistent with this idea, GS cells that were initially established from gonocytes in the presence of LIF could later be maintained in the absence of LIF, suggesting that conversion from gonocyte to spermatogonial stem cells had occurred in the culture.

The results of our experiments raise several questions about the mechanism of GS cell colony formation. We think that at least two factors are important for the success of GS cell culture. One factor is the removal of testicular somatic cells. GS cells do not require somatic cells for their survival and can grow in the complete absence of feeder cells [50, 51]. On the contrary, in the initial phase of the culture, the growth of GS cells is often very slow and is easily overwhelmed by actively proliferating somatic cells. Particularly in pup or adult testis cell culture, since the somatic cells tend to proliferate more actively than the neonatal testes, the establishment of GS cells from these stages requires the highly enriched germ cells. LIF appears to stimulate the proliferation of neonatal somatic cells to some degree, but it does not appear to have an apparent effect on the culture of the pup or adult testis, in which somatic cells proliferate more actively, regardless of LIF. In addition, although we have shown that mouse embryonic fibroblasts have beneficial effects on GS cell growth after their establishment [50], the negative effects of testicular somatic cells on gonocytes and spermatogonia have previously been reported [52, 53]. Therefore, we do not think that LIF acts by promoting the attachment of germ cells to somatic cells.

Another factor is the identification of the appropriate growth factor. It is well established that GDNF is essential for the self-renewal of spermatogonial stem cells. However, it is still unknown when the male germline cells begin to acquire responsiveness to GDNF. Even in the presence of GDNF and LIF, we could not establish the GS cell from the 14.5- to ~16.5-dpc fetal gonocyte, suggesting that gonocytes have different growth requirements, depending on their developmental stage. While LIF may be only one of the molecules that enhance the differentiation of gonocytes to spermatogonial stem cells, there are probably other unknown growth factors that coordinate with LIF to promote the maturation of gonocytes into self-renewing spermatogonia. The identification of these molecules may be required to derive GS cells from different stages of testicular development.

Interestingly, the effect of LIF was mimicked by CNTF, which belongs to the IL-6 superfamily. However, OSM, another member of this family, did not have a similar effect, suggesting that these molecules have differential effects in the testis. Although these IL-6 family molecules are expressed in the neonatal testis, their functions are poorly understood. Previously, OSM was reported to be involved in the regulation of Sertoli cells [28]. The addition of OSM in vitro enhanced the proliferation of Sertoli cells derived from the neonatal testis, but LIF did not exhibit a similar activity [28]. Although both LIF and CNTF were able to enhance the formation of germ cell colonies in the present study, their mechanisms of action are unclear. They have been thought to transmit signals through the IL-6ST receptor, but a recent study showed that the IL-6ST receptor is dispensable in the male germline and that a male lacking IL-6ST retained normal fertility [46]. However, because the deletion efficiency was not assessed in that study, it remains possible that deletion did not occur in all of the male germ cells owing to incomplete cre-mediated deletion. Alternatively, another unknown receptor for LIF or CNTF may compensate for the loss of IL-6ST. Further studies are clearly required to determine which molecules trigger the gonocyte-spermatogonium transition in vivo.

The success of spermatogonia culture has opened up new possibilities in spermatogonia research. While GS cells can be cultured in serum- or feeder-free conditions, they can also be propagated in the absence of substrata [50, 51]. Furthermore, knockout animals can be produced by gene trapping and homologous recombination in GS cells [54], and these cells can potentially convert into pluripotent ES-like cells [55]. The conditions presently used for culture are not optimal, however, and many improvements are needed to increase the usefulness of the technology. In particular, the initiation phase of culture is probably the most critical, because the overgrowth of somatic cells often interferes with the growth of germ cells. We have demonstrated here that LIF is one of the molecules that enhance the initiation phase, but much remains to be studied about the process of GS cell derivation and its relation to male germline development. Our present experimental system may prove valuable for identifying other factors involved in the functional differentiation of PGCs to gonocytes and spermatogonial stem cells.

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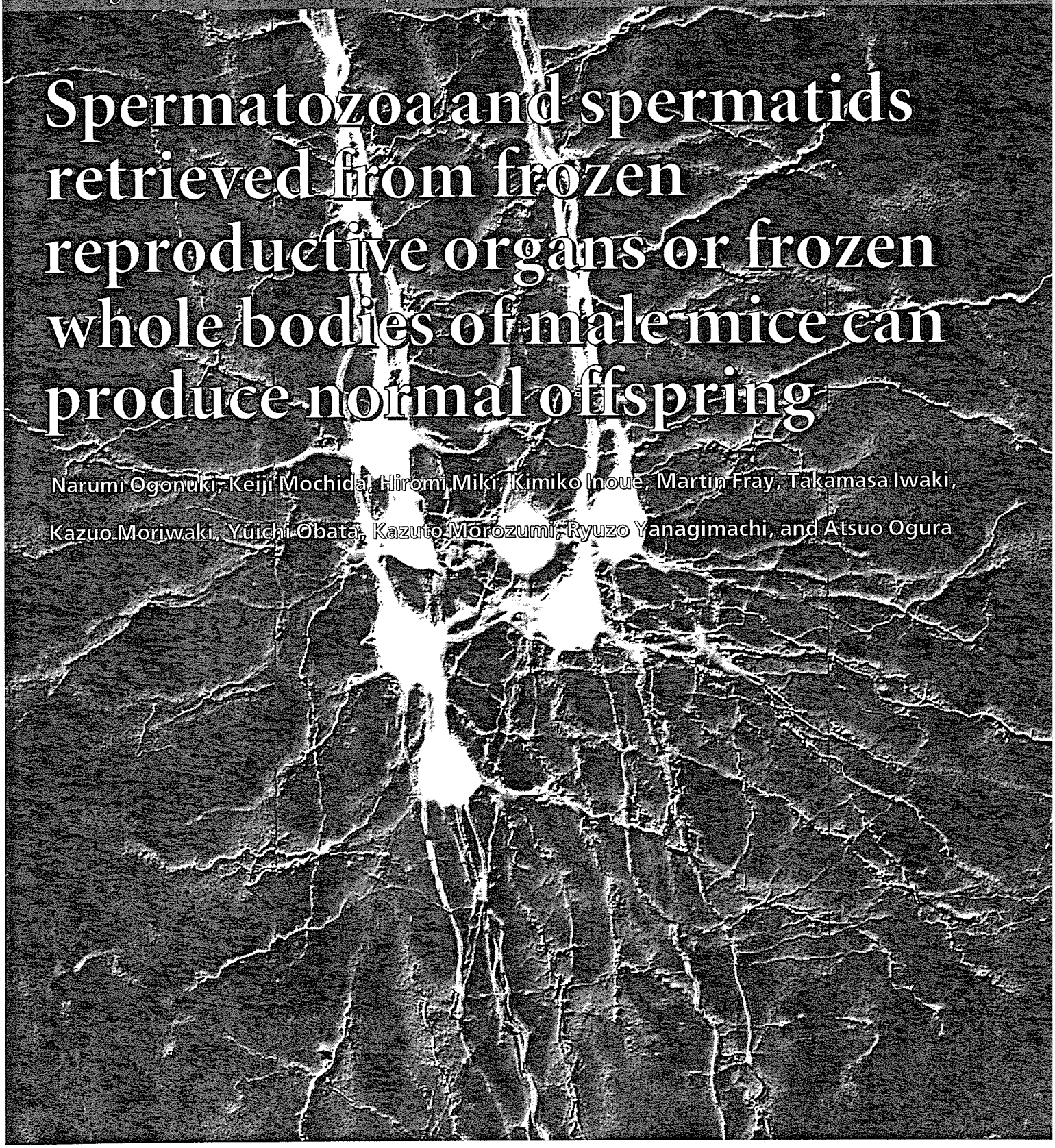

Spermatozoa and spermatids retrieved from frozen reproductive organs or frozen whole bodies of male mice can produce normal offspring

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Contributed by Ryuzo Yanagimachi, July 9, 2006

Cryopreservation of male germ cells is a strategy to conserve animal species and strains of animals valuable to biomedical research. We tested whether mouse male germ cells could be cryopreserved without cryoprotection by simply freezing epididymides, testes, or whole bodies. The reproductive organs were isolated from killed mice and frozen for 1 week to 1 year at −80°C before spermatozoa and spermatids were collected and injected into mature oocytes. Normal pups were born irrespective of strains tested (ICR and C57BL/6). Epididymides and testes frozen and transported internationally to another laboratory by air could produce pups of inbred C57BL/6 mice. Testicular spermatozoa retrieved from the bodies of male mice (BALB/c nude and C3H/He strains) that had been kept frozen (−20°C) for 15 years could also produce normal offspring by microinsemination. Thus, freezing of either male reproductive organs or whole bodies is the simplest way to preserve male germ cells. Restoration of extinct species could be possible if male individuals are found in permafrost.

cryopreservation | gametes | intracytoplasmic sperm injection | microinsemination | mouse

Sperm cryopreservation has been widely used for both human reproduction and animal breeding. Because much of basic research of mammalian genetics and early development has been done by using laboratory mice, and the number of genetically engineered mice (transgenesis, gene targeting, and mutagenesis) is increasing exponentially, development of simple, cost-saving, and space-effective means of mouse sperm preservation is much needed (1). Mouse sperm cryopreservation using raffinose and skim milk as cryoprotectants has been successful, but defrosted spermatozoa of some strains of mice do not fertilize eggs well (2). This is especially true for C57BL/6 (B6) mice, most frequently used for generation of genetically engineered mice. This problem has been overcome by partial zona dissection before *in vitro* fertilization (3) or intracytoplasmic sperm injection of frozen-thawed or freeze-dried spermatozoa (4–7).

Immature male germ cells such as spermatids and spermatocytes are currently used to produce offspring (8–11) when spermatozoa cannot be obtained due to spermatogenic arrest because of genetic mutations or as the result of *in vitro* manipulation of germ cells (7, 10, 11). Therefore, cryopreservation of these immature sperm cells is necessary, but they suffer more cryodamage than mature epididymal spermatozoa. A cryopreservation protocol we developed for mouse spermatogenic cells in the past is rather complicated, and therefore the development of simpler techniques is required (12).

Thus far, the simplest method of cryopreserving mouse spermatozoa is to freeze spermatozoa in simple salt solutions without any cryoprotectants (6). Although defrosted spermatozoa are not alive in the conventional sense, they apparently maintain their genetic integrity, because they are able to produce live offspring by microinsemination. Here we report that spermatozoa or spermatids, retrieved from frozen reproductive organs or frozen bodies of mice, can produce offspring by microinsemination.

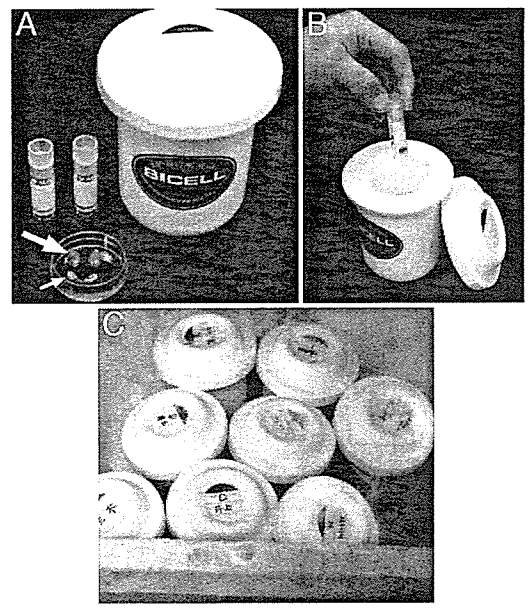

Fig. 1. Freezing epididymides and testes. (*A*) Epididymides (small arrow) and testes (large arrow) removed from adult male mice; two cryotubes (upper left) and a freezing container (Bicell, upper right). (*B*) Epididymides and testes are put in cryotubes before being placed in a freezing container. (*C*) Freezing containers stored in a freezer.

Results

Reproductive Capacity of Spermatozoa and Spermatids in Frozen Epididymides and Testes. First, we examined whether spermatozoa and spermatids within epididymides and testes withstand freezing and thawing. We used ICR mice, because their spermatozoa are known to be less sensitive to damage by freezing and thawing than those of B6 mice (2). Table 1 summarizes the results of experiments in which isolated epididymides and testes were frozen for 1–8 weeks in liquid nitrogen (−196°C) or in freezers (−80°C) with or without using freezing containers (Fig. 1). Spermatozoa retrieved from the epididymides or testes were completely immotile and "dead," as

Conflict of interest statement: No conflicts declared.

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Abbreviations: NIM, nucleus isolation medium; CZB medium, Chatot, Ziomet, and Bavister medium.

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Table 1. Development of embryos after microinsemination of oocytes with male germ cells collected from epididymides and testes that were frozen for 1–8 weeks under various freezing protocols (ICR male germ cells and B6D2F1 oocytes)

Male germ cells	Freezing temperature, °C	Use of freezing container	No. of two-cell embryos transferred	No. of recipient females		Total no. of implantation sites (%)‡	No. of live term fetuses (%)
				Total	Pregnant		
Epididymal sperm	−196*	−	42	3	1	5 (11.9)	1 (2.4)
	−80	−	99	7	0	0 (0.0)	0 (0.0)
	−80	+	73	6	2	19 (26.0)	2 (2.7)
	−80 and −196†	+	63	4	2	8 (12.7)	2 (3.2)
Testicular sperm	−196*	−	35	3	2	13 (37.1)	5 (14.3)
	−80	−	31	3	3	19 (61.3)	10 (32.3)
	−80	+	15	2	2	9 (60.0)	5 (33.3)
	−80 and −196†	+	21	2	2	10 (47.6)	3 (14.3)
Testicular round spermatids	−196*	−	55	4	2	2 (3.6)	1 (1.8)
	−80	−	71	5	5	47 (66.2)	19 (26.8)
	−80	+	94	7	7	61 (64.9)	24 (25.5)
	−80 and −196†	+	65	4	4	48 (73.8)	17 (26.2)

Results of two replicates.
*Direct plunging of epididymides or testes into liquid nitrogen.
†Frozen at −80°C for 1 day, then stored in liquid nitrogen for 1–8 weeks.
‡Live fetuses plus resorption sites.

confirmed by propidium iodide staining (LIVE/DEAD Sperm Viability Kit, Molecular Probes, Eugene, OR; data not shown). Virtually all round and elongated spermatids had extensively disintegrated cytoplasm or no cytoplasm around their nuclei (Fig. 2). In all experiments, >70% of oocytes survived injection of sperm and round spermatids, and >80% of them developed into two-cell embryos, although those injected with round spermatids required exogenous activation stimulus to develop (see *Materials and Methods*). Therefore, we performed statistical analysis of surviving fetuses based on the number of two-cell embryos transferred to recipient females. A statistical analysis (two-way ANOVA) revealed that both cell type and freezing protocol for the cooling rate (see Fig. 3) had significant effects on embryo implantation and fetal development ($P < 0.05$). As seen in Table 1, epididymal spermatozoa produced considerably fewer live offspring than testicular spermatozoa and spermatids [Fisher's protected least significant difference (PLSD); $P < 0.05$]. Direct plunging of testes into liquid nitrogen and subsequent recovery and injection of round spermatids also resulted in poor embryo development (Fisher's PLSD; $P < 0.05$). There was no significant interaction between the two factors (cell type and freezing protocol) with respect to both implantation rates and birth rates ($P > 0.05$), indicating that these factors are independent of each other.

Second, we studied whether spermatozoa and spermatids within epididymides and testes of B6 males withstand freezing and thawing. We used freezing containers for this experiment, because they gave good results in the previous experiments (Table 1). Oocytes were collected from B6 females to maintain inbred background of offspring. There was a significant effect of cell type on both implantation and birth rates ($P < 0.05$; one-way ANOVA). Results, summarized in Table 2, indicated that spermatozoa collected from frozen cauda epididymis produced live offspring when defrosted

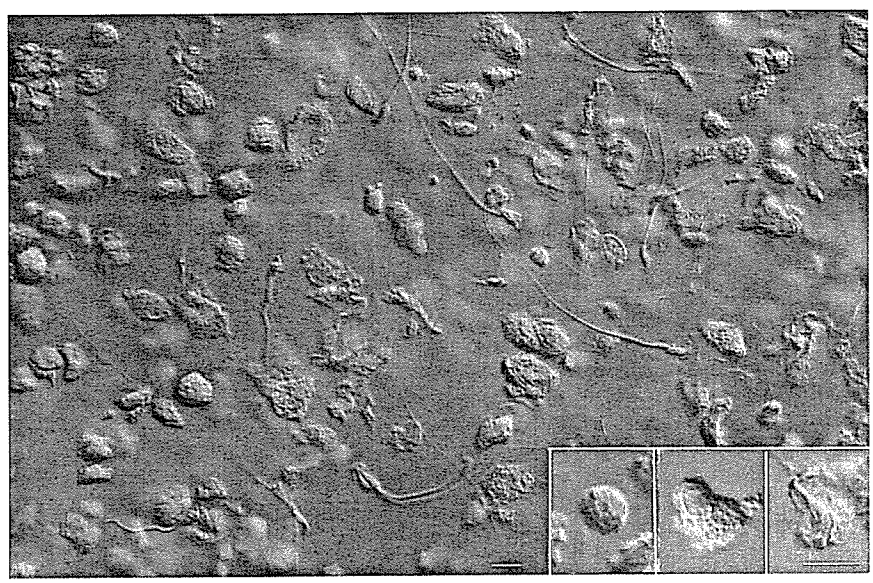

Fig. 2. Spermatozoa and spermatogenic cells collected from a testis frozen at −80°C for 1 month. Haploid germ cells can be identified by their size and nuclear shape, although their plasma membrane is disintegrated extensively. (*Insets*, from the left) A presumptive round spermatid, an early elongated spermatid, and an elongated spermatid. (Bar, 10 μm.)

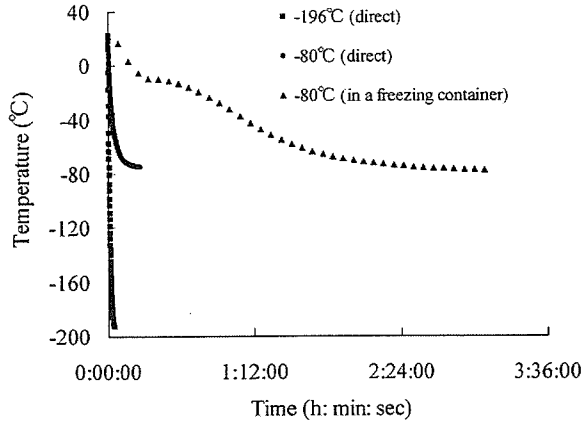

Fig. 3. Temperature changes inside cryotubes in different freezing protocols. Temperature declines most slowly when the cryotube is placed in a freezing container and is fastest when a cryotube is plunged directly into liquid nitrogen (LN_2).

spermatozoa were suspended in K^+-rich nucleus isolation medium (NIM) (13), not conventional GL-PBS medium (Dulbecco's PBS supplemented with 5.6 mM glucose/5.4 mM sodium lactate/5 mg/ml BSA/0.01% polyvinyl alcohol; ref. 12), before injection into oocytes ($P < 0.05$; vs. suspension in GL-PBS). Spermatozoa and round spermatids collected from frozen testes were able to produce offspring without using NIM.

International Transportation of Frozen Testes. We tested whether frozen epididymides and testes of B6 mice can be transported internationally by shipping frozen samples with dry ice from the United Kingdom to Japan by air. Spermatozoa and elongated spermatids released from defrosted epididymides and testes into GL-PBS or NIM were injected into oocytes. As shown in Table 3, normal live B6 offspring were obtained in all test groups except for epididymal spermatozoa suspended in GL-PBS. NIM always gave better results than GL-PBS.

Long-Term Storage of Male Germ Cells in Frozen Testes or Whole Bodies. In the last series of experiments, we tested the feasibility of long-term storage of frozen testes or frozen bodies. When ICR male germ cells were retrieved from testes frozen at −80°C for 1 year, suspended in NIM, and injected into oocytes, normal pups were born at moderate rates (11.5–22.6%; Table 4). Although the spermatozoa retrieved from mice frozen for 15 years looked severely damaged (Fig. 4*A*), heads could be separated from tails as easily as those of fresh live spermatozoa. Within 24 h after sperm injection, >80% of oocytes developed into two-cell embryos, and apparently normal pups were born after embryo transfer in two strains of mice (BALB/c-nude and C3H/He) at fairly high rates (Table 5). Two BALB/c pups died shortly after Caesarian section due to respiratory failure but others grew normally (Fig. 4*B*) and were proven to be fertile when they matured (at least 19 of 20 mice tested).

Discussion

We reported here that the spermatozoa and spermatids collected from frozen epididymis and testis or from frozen bodies of mice were able to produce normal offspring after injection into oocytes. Obviously, those frozen spermatozoa and spermatids were all "dead" in the conventional sense, as confirmed by propidium iodide staining. Their plasma membranes were severely damaged. Motionless spermatozoa had no chance whatsoever of fertilizing oocytes *in vivo* or *in vitro*. Nevertheless, some of these spermatozoa and spermatids, if not all, were "alive" or "genomically intact," because they were able to produce apparently normal offspring.

Freezing protocols used here are very simple. Simple freezing of animal bodies, using ordinary freezers, is perhaps the simplest procedure we can think of, and it can be done in any laboratory. Isolation and freezing of testes and epididymides are also simple and can be done in most laboratories or even in the field. Our protocols require a set of micromanipulators to generate offspring, but microsurgical injection of spermatozoa (or spermatogenic cells) into oocytes is no longer a special procedure. In fact, thousands of infertility clinics throughout the world routinely perform microsurgical sperm injection to overcome various types of male infertility (14, 15). Normal births after microinsemination have been reported in 14 mammalian species, as of today (11). Laboratories that do not have microsurgical facilities or experience may keep the frozen testis/epididymis or entire bodies in an ordinary freezer (without an automatic defrosting cycle) until they are ready to send the specimens to other laboratories, where microinsemination is routinely performed. This would be particularly pertinent when precious male animals died unexpectedly.

Of all mouse strains, C57BL/6 (B6) would receive the most benefit from this freezing procedure. B6 mice have been most extensively used as a standard strain of mouse for genetic studies. Many genetically modified mice have a B6 background. Difficulty in cryopreserving their spermatozoa has been a major obstacle in mouse genetics (2, 16). Here we demonstrated that B6 mice can be produced by using spermatozoa retrieved from testes frozen for 5–7 months or air-transported with dry ice. The freezing protocols reported here are simple and cost-effective. They would enhance exchange of mouse genetic resources among many laboratories around the world.

It was rather unexpected that spermatozoa in the testes would withstand freezing better than those in the epididymis (Table 3), because nuclei of epididymal spermatozoa are known to be much more stable, both physically and chemically, than testicular spermatozoa because of extensive crosslinkings of nuclear protamines by disulfide bonds (17, 18). Our two-factorial statistical analysis, consisting of three germ cell types and four freezing protocols (Table 1), revealed that epididymal spermatozoa were most sensitive to freezing of all germ cells examined. The reason for this is not clear, but luminal fluid in the seminiferous tubule and/or some materials from Sertoli or spermatogenic cells might

Table 2. Development of embryos after microinsemination of oocytes with male germ cells collected from caudae epididymides and testes that were frozen at −80°C for 5–7 months (B6 male germ cells and B6 oocytes)

Male germ cells (postthawing medium)	No. of two-cell embryos transferred	No. of recipient females		Total no. of implantation sites (%)	No. of normal term fetuses (%)
		Total	Pregnant		
Epididymal sperm (GL-PBS)	63	5	0	0 (0.0)*	0 (0.0)†
Epididymal sperm (NIM)	73	8	6	43 (58.9)**	17 (23.3)††
Testicular sperm (GL-PBS)	26	2	2	10 (38.5)	5 (19.2)††
Testicular round spermatids (GL-PBS)	50	5	4	26 (52.0)**	13 (26.0)††

Results of two replicates. * vs. ** and † vs. ††, $P < 0.05$; Fisher's protected least significant difference test.

Table 3. Development of embryos after microinsemination of oocytes with spermatozoa and spermatids from frozen epididymides and testes, transported internationally (B6 male germ cells and B6 oocytes)

Male germ cells	Cells suspension medium	No. of two-cell embryos transferred	No. of recipient females		Total no. of implantation sites (%)	No. of normal term fetuses (%)
			Total	Pregnant		
Epididymal sperm	GL-PBS	63	5	0	0 (0.0)*	0 (0.0)‡
	NIM	38	3	2	17 (44.7)**	9 (23.7)‡‡
Testicular sperm	GL-PBS	50	4	4	34 (68.0)†	11 (22.0)§
	NIM	20	2	2	19 (95.0)††	13 (65.0)§§
Testicular elongated spermatids	GL-PBS	10	1	1	3 (30.0)	1 (10.0)
	NIM	39	4	3	12 (30.8)	7 (17.9)

*, **, $P < 5 \times 10^{-8}$. †, ††, $P < 0.05$. ‡, ‡‡, $P < 1 \times 10^{-4}$. §, §§, $P < 1 \times 10^{-3}$ (Fisher's exact probability test). ‡, ‡‡, $P < 1 \times 10^{-4}$. §, §§, $P < 1 \times 10^{-3}$ (Fisher's exact probability test).

have contributed to alleviating sperm damage by freezing. Although round spermatids recovered from defrosted testes can be used to produce live offspring (Tables, 1, 2, and 4), selective identification of these cells from other cells could be rather difficult for inexperienced researchers. Elongated spermatids or spermatozoa are obviously much easier to identify (Fig. 2).

One thing to be stressed here is that the type of medium used for suspension of defrosted spermatozoa and spermatids makes a difference in the outcome of the experiments: the birth of normal offspring. Potassium-rich Ca^{2+}- and Mg^{2+}-free NIM medium (13, 19) always gave better results (Tables 2 and 3). Ca^{2+}-containing ordinary cell culture media like PBS may activate endogenous nucleases (20), which attack the DNAs of these spermatozoa and spermatids with broken plasma membranes. Cauda epididymal spermatozoa may have a higher nuclease activity than testicular spermatozoa and spermatids, and this may make the former more sensitive to freezing and thawing than the latter.

The present study has shown that spermatozoa and spermatids can retain their fertilizing ability in frozen reproductive organs or whole bodies for longer than we anticipated. We found that the spermatozoa retrieved from the testes of mice frozen at −20°C for 15 years were able to produce normal offspring by microinsemination. It would be interesting to know the optimal temperature to use for whole-body freezing and how long male germ cells can retain their fertilizing ability. Accelerated degradation kinetics that have been applied to estimate the maximum storage period of freeze-dried mouse spermatozoa (21) may be applicable to answer this question. If spermatozoa of extinct mammalian species (e.g., woolly mammoth) can be retrieved from animal bodies that were kept frozen for millions of years in permanent frost, live animals might be restored by injecting them into oocytes from females of closely related species.

Materials and Methods

Preparation of Mouse Oocytes. Female B6D2F1 and C57BL/6 (B6) mice (7–10 weeks old) were each injected with 7.5 units of equine chorionic gonadotropin followed by injection of 7.5 units of human chorionic gonadotropin (hCG) 48 h later. Mature oocytes were collected from oviducts 15–17 h after hCG injection and were freed from cumulus cells by a 3-min treatment with 0.1% hyaluronidase in Chatot, Ziomet, and Bavister (CZB) medium (22). The oocytes were transferred to fresh CZB medium and incubated in it at 37°C in an atmosphere of 5% CO_2 in air for up to 90 min before micromanipulation.

Cryopreservation of Isolated Epididymides and Testes. Caudae epididymides and testes were isolated from sexually mature male ICR and B6 mice (2–6 months of age), and each was placed at the bottom of a 2-ml polypropylene cryotube (12.5 × 48 mm; MS-4503, Sumitomo Bakelite, Tokyo, Japan; Fig. 1*A*). Some tubes were plunged into liquid nitrogen and kept there. Other tubes were transferred into a deep freezer (−80°C) with or without a freezing container (Bicell; Nihon Freezer, Osaka, Japan; Fig. 1 *B* and *C*). Some tubes, frozen in a freezing container, were transferred to and kept in liquid nitrogen. After storage for 1 week to 1 year, the cryotubes with frozen epididymides and testes were put in an 1-liter water bath (25°C) for ≈2 min. It was important to collect spermatozoa and spermatids immediately after defrosting.

Collection of Spermatozoa and Spermatogenic Cells from Defrosted Epididymis and Testis. A defrosted cauda epididymis was placed under mineral oil in a Petri dish and punctured with a 26-gauge needle to release spermatozoa. Using a 200-μl micropipette, a sperm mass was transferred to the bottom of a 1.5-ml plastic centrifuge tube and gently covered with 200 μl of GL-PBS (12). After gentle pipetting, the sperm suspension was kept at 4°C. In some experiments, spermatozoa were suspended in K^+-rich NIM medium. Testicular spermatozoa and spermatids were mechanically isolated from defrosted testes, as described for hamsters (23). Defrosted testes were placed in erythrocyte-lysing buffer (155 mM NH_4Cl/10 mM $KHCO_3$/2 mM EDTA, pH 7.2), and the tunica albuginea were removed. Seminiferous tubules were transferred into cold (4°C) GL-PBS and cut into small pieces using a pair of fine scissors. They were gently pipetted to allow spermatogenic cells to disperse into the medium. The cell suspension was filtered through a 38-μm nylon mesh and washed twice by centrifugation (200 × *g*

Table 4. Development of embryos after microinsemination of oocytes with male germ cells collected from the testes kept at −80°C for 1 year (ICR male germ cells and B6D2F1 oocytes)

Male germ cells	No. of two-cell embryos transferred	No. of recipient females		Total no. of implantation sites (%)	No. of normal term fetuses (%)*
		Total	Pregnant		
Testicular sperm	31	2	2	16 (51.6)	7 (22.6)
Elongated spermatids	32	2	2	9 (28.1)	6 (18.8)
Round spermatids	61	3	3	26 (42.6)	7 (11.5)

*The results within the same column were not significantly different ($P > 0.05$, Fisher's exact probability test).

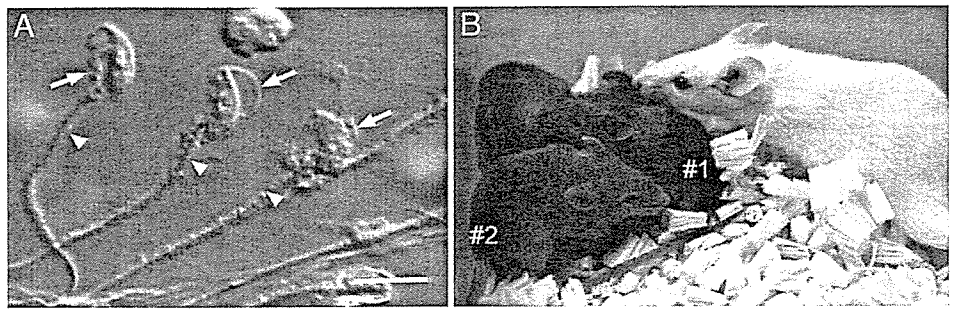

Fig. 4. Production of mice using spermatozoa retrieved from testes of male mice frozen for 15 years. (*A*) Spermatozoa collected from seminiferous tubules show severe structural damages. The head (arrows) and midpiece (arrowheads) have cellular debris. (*B*) Pups born after injection of frozen spermatozoa (BALB/c-nude) into B6D2F1 oocytes. Because BALB/c and B6D2F1 have the coat-color genotype of A/A b/b c/c D/D and A/a B/b C/C D/D, respectively, the offspring are agouti (heterozygous for all loci; #1) or brown (homologous for the b locus; #2). The albino mouse is their foster mother.

for 4 min). After gentle washings, cells were resuspended in GL-PBS and were stored at 4°C for up to 120 min.

Cryopreservation and Thawing of Whole Mouse Bodies. Mature males of BALB/c-nude and C3H/He strains (8 weeks of age) were killed by overdose of pentobarbital on February 20, 1991 and March 8, 1991, respectively, and kept in a −20°C freezer without automatic defrost cycle. The body of a BALB/c-nude mouse was thawed on February 6, 2006, and that of a C3H/He mouse on February 13, 2006, by putting them in a large water bath (>3 liters of water at 25°C) for 5 min until the outer surface of the body was softened. The body was then removed from the water, and the testes were isolated through an abdominal incision. Germ cells were released from the testes, as already described.

International Transport of Frozen Epididymides and Testes. Epididymides and testes were collected from B6 mice (3 months old), and each was placed in a cryotube. Several cryotubes, placed in a freezing container, were stored in a deep freezer (−80°C) for 1 day. Immediately before shipment, cryotubes were put in a styrene foam box filled with dry ice and sent by air from Oxfordshire, U.K., to Tsukuba, Japan. After 3 days of air transportation, the cryotubes were transferred to and kept in a deep freezer (−80°C) for ≈1 month before thawing.

Microinsemination. Intracytoplasmic injection of spermatozoa and spermatids was performed by using a micropipette attached to a Piezo-electric actuator (Prime Tech, Ibaraki, Japan), as described (24, 25). The cover of a plastic dish (50 × 3 mm; Falcon no. 1006; Becton Dickinson, Franklin Lakes, NJ) was used as a microinjection chamber. Several small drops (≈2 μl) of Hepes-CZB with or without 10% polyvinylpirrolidone (PVP) were placed on the bottom and covered with mineral oil. Spermatozoa and spermatogenic cells were placed in one of the PVP droplets. A single spermatozoon was sucked, tail first, into an injection pipette, and the head was separated from the tail by applying a few Piezo pulses to the head–tail junction. The isolated sperm head was injected into an oocyte in Hepes-buffered CZB (24). Before injection of the nuclei of round spermatids, oocytes were activated by treatment with Ca^{2+}-free CZB medium containing 2.5 mM $SrCl_2$ for 20 min at 37°C. The oocytes, at 45–90 min after onset of activation treatment (reaching telophase II), were each injected with a round spermatid. Operated oocytes were kept in Hepes-CZB at room temperature (25°C) for ≈10 min before culture in CZB at 37°C under 5% CO_2 in air.

Embryo Culture and Transfer. Embryos that reached the two-cell stage by 24 h of culture in CZB were transferred into the oviducts of pseudopregnant ICR females (8–12 weeks old) on the day after mating (day 0.5). On day 19.5, recipient females were killed, and their uteri were examined for live term fetuses. In some experiments, live fetuses were nursed by lactating ICR females to see whether they could survive until weaning.

Care and Use of Animals. All procedures described here were reviewed and approved by the Animal Experimental Committee at the RIKEN Institute and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Statistical Analysis. The efficiencies of embryo development were analyzed by using arcsine transformation, followed by two-way ANOVA analysis. A post hoc procedure using Fisher's protected least significant difference test was adopted for multiple comparisons where appropriate. Results were evaluated by using Fisher's exact probability test when each experimental group consisted of a single experiment (Tables 3 and 4). A P value <0.05 was considered statistically significant.

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Table 5. Development of embryos after microinsemination with spermatozoa collected from testes of mice frozen at −20°C for 15 years (B6D2F1 oocytes)

Strain of male mouse	No. of oocytes that survived injection	No. of oocytes cleaved (%)	No. of two-cell embryos transferred	No. of recipient females		Total no. of implantation sites, (%)	No. of normal term fetuses (%)	No. weaned (%)
				Total	Pregnant			
BALB/c-nude	106	81 (76.4)	81	6	4	42 (51.9)	17 (21.0)	15 (88.2)
C3H/He	108	97 (89.8)	97	7	4	34 (35.1)	12 (12.4)	12 (100.0)

We could not use elongated and round spermatids from testes because of the complete degeneration of these cells.

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