Healthy offspring from freeze-dried mouse spermatozoa held on the International Space Station for 9 months

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If humans ever start to live permanently in space, assisted reproductive technology using preserved spermatozoa will be important for producing offspring; however, radiation on the International Space Station (ISS) is more than 100 times stronger than that on Earth, and irradiation causes DNA damage in cells and gametes. Here we examined the effect of space radiation on freeze-dried mouse spermatozoa held on the ISS for 9 mo at –95 °C, with launch and recovery at room temperature. DNA damage to the spermatozoa and male pronuclei was slightly increased, but the fertilization and birth rates were similar to those of controls. Next-generation sequencing showed only minor genomic differences between offspring derived from space-preserved spermatozoa and controls, and all offspring grew to adulthood and had normal fertility. Thus, we demonstrate that although space radiation can damage sperm DNA, it does not affect the production of viable offspring after at least 9 mo of storage on the ISS.

International Space Station | preservation | freeze-dry | spermatozoa | fertilization

Since the "space dog" Laika (Лайка) was first placed into orbit in 1957 (1), many humans and animals have been to space or stayed on the International Space Station (ISS) for more than 6 mo. In the future, humans likely will live on large-scale space stations or in other space habitats for several years or even over many generations. At that time, assisted reproductive technology (ART) likely will be used to produce humans in space habitats, given that the use of ART by infertile couples has increased year by year and that ART can be performed with cryopreserved spermatozoa or embryos (2, 3). In a similar way, domestic animals likely will be generated by artificial insemination (AI) in space, because many domestic animals are already produced by AI using long-term cryopreserved spermatozoa (4). In addition, genetic diversity is very important for maintaining a species, especially in small colonies, and this could be achieved by cryopreserving a diverse range of gamete cells. The environment in space is very different from that on Earth, however, including high levels of space radiation and microgravity, and the effects of these factors on mammalian reproduction are largely unknown. Although with current technology, producing offspring in such an environment can be difficult or dangerous (5), the study of reproduction in space is a very important subject for our future.

So far, the effects of microgravity on early development have been studied using sea urchins, fish, amphibians, and birds (6–12). These studies have concluded that microgravity does not prevent animal reproduction. However, because of the difficulty in maintaining mammals and performing experiments in space, studies of mammal reproduction in space have not progressed as well as in other animals, and only a few papers have been published (13–18). Those studies and our previous study (19) have suggested that mammalian reproduction in space under conditions of microgravity cannot be easily compared with reproduction in other species.

Another difference between space and Earth is the high level of radiation in space. The doses received inside the ISS depend on sunspot cycles and cosmic rays. The average dose rate measured at the ISS is ∼0.5 mSv/day, roughly 100-fold higher than that measured on Earth (20). Ground-based studies have demonstrated the deleterious effects of radiation on living organisms, including the induction of mutations and tumor formation (21, 22). Humans living for several generations in space habitats or traveling to Mars will encounter much higher cosmic radiation, possibly putting them at high risk for cancer (23). If space radiation also causes DNA damage to cryopreserved spermatozoa, it might lead to serious problems, such as embryo death or abortion, or cause mutations in offspring and subsequent generations.

Salamander and medaka fish eggs could be fertilized and developed normally during orbital space flight (6, 24), suggesting that space radiation from brief space flights does not affect fertilization or later embryogenesis in these vertebrates. However, mammalian oocytes are known to have a strong potential for

Significance

Radiation on the International Space Station (ISS) is more than 100 times stronger than at the Earth's surface, and at levels that can cause DNA damage in somatic cell nuclei. The damage to offspring caused by this irradiation in germ cells has not been examined, however. Here we preserved mouse spermatozoa on the ISS for 9 mo. Although sperm DNA was slightly damaged during space preservation, it could be repaired by the oocyte cytoplasm and did not impair the birth rate or normality of the offspring. Our results demonstrate that generating human or domestic animal offspring from space-preserved spermatozoa is a possibility, which should be useful when the "space age" arrives.

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repairing damaged DNA (25–27), so when live animals are in space for only short periods, the effect of space radiation might be masked by subsequent repair of the damaged DNA after fertilization. In contrast, although cryopreserved cells are still alive, they have stopped metabolizing, which means that they cannot repair DNA damage while still frozen (28, 29). Therefore, DNA damage might accumulate with increased duration in space. If this is so, then the production of offspring from long-term cryopreserved spermatozoa in space will be compromised because of increased embryo mortality or mutation rates in the offspring. Therefore, it is very important to examine the effect of space radiation on spermatozoa preserved in space. In addition, the resistance of spermatozoa likely differs among species, because the sperm structure differs (30). Therefore, to examine the influences of radiation in mammalian species, we must use mammalian species, and the mouse is a very convenient model animal for space study.

For the present study, we decided to freeze-dry the mouse spermatozoa (31) rather than use traditional cryopreservation methods. When spermatozoa are freeze-dried or evaporatively dried, none of the sperm survive; however, mouse spermatozoa can maintain the ability to generate offspring when added to water and microinjected into fresh oocytes (31, 32), and this may possible with human spermatozoa in the future (33, 34). More importantly, such dried spermatozoa can be preserved at room temperature for up to 2 y (35, 36) and in a freezer almost indefinitely (37). In addition, the samples are very light and occupy a small volume. Therefore, our samples could be launched to the ISS without the need for a freezer, which greatly reduced the cost of launching. The merits of this procedure, in terms of the ease of launching freeze-dried samples into space, are significant for enabling the study of mammalian reproduction in space, even though the production rate of offspring from freeze-dried spermatozoa is lower than that from traditional cryopreservation methods.

After exposure to space radiation for 9 mo at –95 °C, the samples were returned to Earth. We evaluated these samples for sperm morphology and DNA damage, capacity for fertilization by microinjection, in vitro developmental potential, and normality of offspring derived from the spermatozoa.

Results

Collection of Space Sperm Samples. The samples (Fig. 1 *A* and *B*) were launched to the ISS on August 4, 2013, and returned to ground on May 19, 2014. Therefore, these space sperm samples were exposed to cosmic radiation for 288 d. The majority of the glass ampules sustained no damage during the launch or return. The ground control sperm samples from the same mice were exposed to the same temperature changes at the same times and for the same durations as the space sperm samples.

Total Doses of Space Radiation. The CR-39 plastic nuclear track detectors (PNTDs) in the Bio PADLES packages collected from the space-preserved cases (Fig. 1*C*) can detect nuclear tracks. Fig. 1*D* shows images of the etch pits corresponding to the tracks

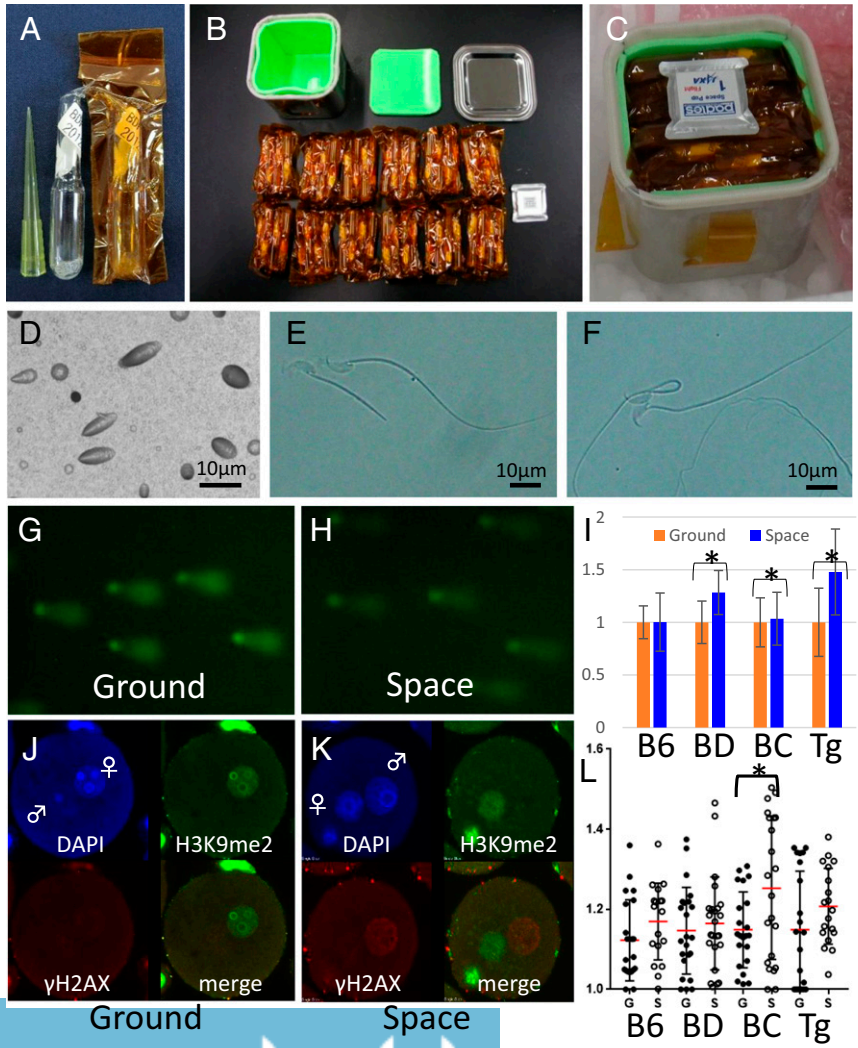

Fig. 1. Preservation of spermatozoa on the ISS and assessment of DNA integrity after return to Earth. (*A* and *B*) Ampules of freeze-dried spermatozoa were wrapped with polyimide film, and then four ampules from each donor mouse were wrapped together. Twelve groups of ampules derived from 12 male mice were selected for this study. The small white square (right side of *B*) represents the PADLES monitor, used to detect the irradiation dose. (*C*) All ampules were inserted into a small case, and a PADLES radiation monitor was placed on top of the case. (*D*) The etch pits corresponding to the tracks of atomic nuclei produced during space flight. (*E* and *F*) Observation of ground control (*E*) and space-preserved spermatozoa (*F*) by light microscopy. (*G–I*) Comet DNA breakage assays of ground control (*G*) and space-preserved spermatozoa (*H*). The lengths of DNA in the comet tails were standardized against the mean lengths of ground control sperm results for each mouse strain (*I*). The orange bars represent the mean lengths of ground control sperm samples after standardization, and blue bars indicate the space sperm samples. The asterisk denotes significant differences between samples (*$P < 0.001$). (*J* and *K*) Immunostaining of zygotes derived from ground control sperm samples (*J*) or space sperm samples (*K*) by the anti–gamma-H2AX antibody. Both male and female pronuclei were detected by nuclear staining with DAPI (*Upper Left*, blue). Female pronuclei were detected by H3K9me2 immunostaining (*Upper Right*, green). The foci of gamma-H2AX signals show DNA double-strand breaks (*Lower Left*, red), and merged images (*Lower Right*). (*L*) The brightness of each male pronucleus was plotted. Black circles indicate zygotes derived from ground control sperm samples; white circles, zygotes derived from space sperm samples. The brightness of the male pronucleus in *K* was 1.3. In *I* and *L*, mouse strains: B6, C57BL/6N; BD, B6D2F1; BC, B6C3F1; Tg, 129B6F1 expressing GFP. Asterisks indicate significant differences (*I*, *$P < 0.001$; *L*, *$P < 0.05$).

of atomic nuclei produced during space flight. Background doses measured in the ground control sperm sample case were subtracted from the net doses measured during space flight. From the results, the absorbed radiation dose rate was calculated as 0.41 ± 0.01 mGy/day, or a dose-equivalent rate of 0.62 ± 0.03 mSv/day, and the total absorbed dose was 117.24 ± 3.98 mGy in water, or a total dose equivalent of 178.35 ± 7.27 mSv.

Morphology and DNA Damage in Spermatozoa. The morphology of space sperm samples could not be distinguished from that of the ground control samples, at least at the light microscopy level (Figs. 1 *E* and *F*). Some spermatozoa showed breakage between the head and tail or fragmented tails, but this is typical for freeze-dried spermatozoa. However, measurement of the comet DNA tails revealed significantly longer tails in the space sperm samples compared with the ground control sperm samples in all mouse strains except the C57BL/6 strain (Fig. 1 *G–I* and Table S1).

In Vitro Fertilization and DNA Damage in Male Pronuclei. When samples were rehydrated, the spermatozoa were used for intracytoplasmic sperm injection (ICSI) of fresh oocytes within 1 h. Most of the oocytes were fertilized and formed normal-appearing pronuclei irrespective of mouse strain, similar to the results for the ground control sperm samples (Table 1). The two-cell rates in this study were slightly lower than those obtained using fresh spermatozoa (~100% in our laboratory).

We next examined the DNA damage in male pronuclei. When zygotes fertilized with space sperm samples were immunostained with the anti–gamma-H2AX antibody, numerous foci were detected in male pronuclei (Fig. 1 *J–L*). Given the difficulty in counting the number of foci inside pronuclei, we measured the brightness of the whole male pronucleus, which was then subtracted from the brightness of the zygote cytoplasm. As shown in Fig. 1*L* and Table S2, the brightness of the male pronuclei varied among zygotes. Although on average, male pronuclei derived from space sperm samples were slightly brighter than those from ground control sperm samples, there were no statistically significant differences between them in any mouse strain except the BCF1 strain.

Developmental Potential in Vitro. To observe the developmental potential of zygotes fertilized with space spermatozoa, some zygotes were cultured in vitro until they developed to the blastocyst stage at 4.5 d after ICSI. Most of the injected oocytes fertilized normally, and some developed into blastocysts (Fig. 2 *A–D*). Some of these blastocysts were immunostained to evaluate the quality based on the cell number and allocation of inner cell mass (ICM) cells. The allocation of ICM cells did not show any clear differences between ground control and space sperm samples (Fig. 2 *E* and *F*). On the other hand, although the mean number of ICM cells was similar in the space and ground control samples (11 and 13, respectively), the mean number of trophectoderm (TE) cells was lower in the space samples (35 vs. 55). However, most of the embryos were used for embryo transfer, and thus only six blastocysts from the space samples and three blastocysts from the ground control samples were examined.

Full-Term Development and Normality of Offspring. The potential for development to full term is the strongest evidence of the normality of the DNA of the space sperm samples. Therefore, when embryos reached the two-cell stage, some were transferred into recipient females, and the birth rates were compared between the space and ground control sperm samples, as well as between the freeze-dried spermatozoa and the original spermatozoa (before preservation). As shown in Table 1 and Fig. 2*G*, the mean birth rates of offspring derived from space sperm samples ("space pups"; Fig. 2*H*) and ground control sperm samples were almost the same (8–17% and 7–15%, respectively) but significantly lower than that with the original spermatozoa (28–39%). The sex ratio of space pups (53% male, 47% female) was within the normal range. After the pups grew to adulthood, two or three pairs from all strains were selected at random and mated with each other. All of these couples had offspring, clearly demonstrating normal fertility.

Next-Generation Sequencing Analysis. Finally, we analyzed the global gene expression profiles of pups derived from space and ground control sperm samples to assess for any nonphenotypic differences. Seven pups (four females, IDs S2, S4, S5, and S7, and three males, IDs S1, S3, and S6) from space sperm samples and six pups (one female, ID G1, and five males, IDs G2–G6) from ground control sperm samples derived from the same male mouse (C57BL/6N strain) were used, and freshly collected brains were examined. From the results of heat mapping (Fig. 2*I*), although the two sets appeared slightly dissimilar, the differences were not statistically significant. There was also no significant difference in gene expression profiles between male and female offspring.

Discussion

In this study, freeze-dried mouse spermatozoa were held on the ISS for 9 mo at –95 °C and then examined after exposure to space radiation.

During the study period, irradiation from space to the sample case was 0.4 mGy/day, or 0.6 mSv/day, which is typical for the ISS and ~100 times higher than that on Earth (29). No special events occurred in the local space during the study period. In these

Table 1. Production of offspring from ground or space-preserved spermatozoa injected into BDF1 oocytes

Mouse strain	Condition of preservation	No. of oocytes	No. of oocytes surviving after ICSI	No. (%) of fertilized embryos	No. (%) of two-cell embryos at 24 h	No. of transferred embryos (no. of recipients)	No. (%) of offspring
C57BL/6	Original	111	89	74 (83)	68 (76)	68 (4)	20 (29)*
	Ground	267	216	208 (96)	165 (76)	165 (8)	19 (12)**
	Space	452	367	359 (98)	262 (71)	262 (12)	24 (9)**
BDF1	Original	110	98	98 (100)	74 (76)	74 (4)	23 (31)*
	Ground	572	423	366 (87)	278 (66)	278 (14)	10 (4)**
	Space	345	282	256 (91)	182 (65)	182 (10)	15 (8)**
BCF1	Original	78	49	48 (98)	38 (78)	38 (3)	16 (42)*
	Ground	270	242	231 (95)	213 (88)	213 (10)	27 (13)**
	Space	400	341	327 (96)	279 (82)	279 (12)	24 (9)**
129B6F1-GFP	Original	102	75	75 (100)	55 (73)	55 (3)	17 (31)*
	Ground	180	140	133 (95)	97 (69)	97 (4)	15 (15)**
	Space	120	86	75 (87)	61 (71)	61 (3)	10 (10)**

Original: When samples were prepared, some ampules were used to check the quality of each lot. These data were used as the original control. Ground: At the same times and for the same durations as when launching and storing the sample case, a control sample case was exposed to room temperature, then frozen at –95 °C and stored in a freezer at the Tsukuba Space Center in Japan. These are referred to as ground control sperm samples. Space: Samples were launched and stored on the ISS for 9 mo. (* vs. **: $P < 0.05$.)

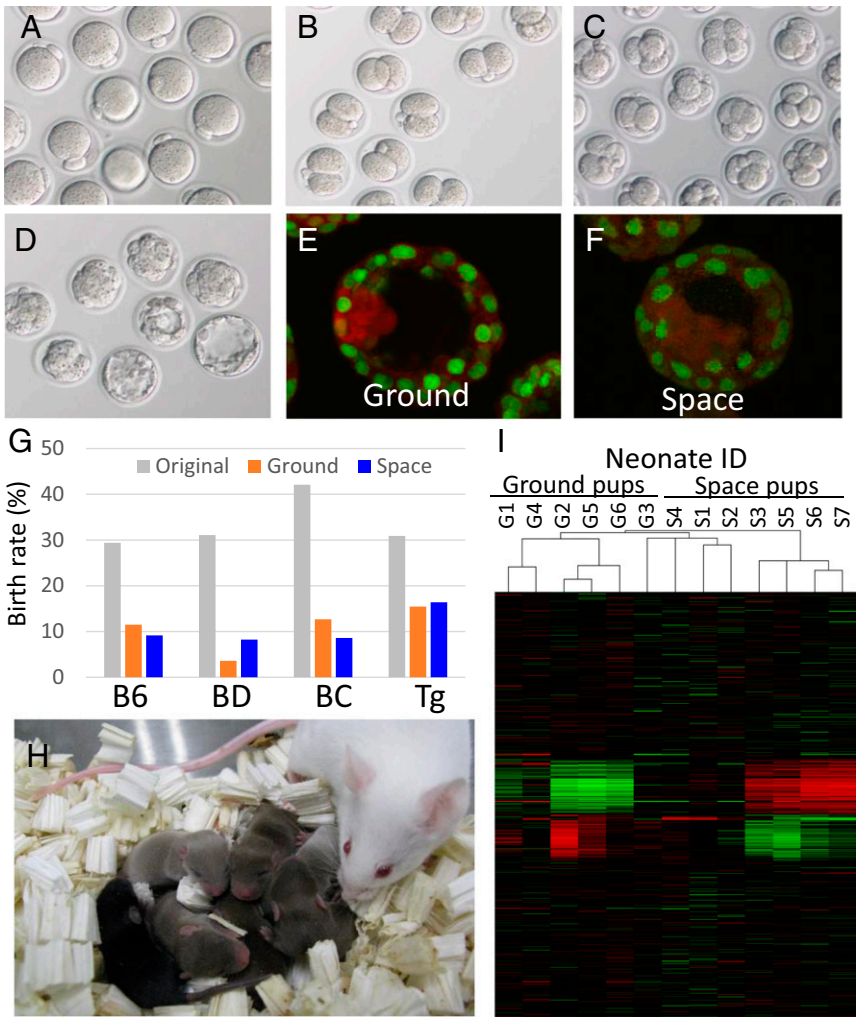

Fig. 2. Developmental potential of embryos. (*A–D*) In vitro development of embryos fertilized by ICSI with space sperm samples. (*E* and *F*) Immunostaining of blastocysts fertilized with ground control (*E*) and space sperm (*F*) samples. $CDX2^{+}$ cells (trophectoderm; TE) are shown in green, and $Oct4^{+}$ cells (inner cell mass; ICM) are in red. (*G*) Full-term development of embryos fertilized with original, ground control, or space-preserved spermatozoa. Gray, orange, and blue bars represent the mean birth rates of embryos derived from original, ground control, and space sperm samples, respectively. Mouse strains: B6, C57BL/6N; BD, B6D2F1; BC, B6C3F1; Tg, 129B6F1 expressing GFP. (*H*) Space pups derived from space-preserved spermatozoa. (*I*) The results of RNA-seq analysis shown as heat maps. Six neonates derived from ground control sperm samples (G) and seven neonates derived from space sperm samples (S) are denoted G1–G6 and S1–S7, respectively.

conditions, our samples were preserved for 288 d and received a total irradiation dose of 117 mGy, or 178 mSv. Previous ground-based studies have suggested that sperm DNA damage occurs when live mice are irradiated with >400 mGy (38, 39), or more than twice the irradiation dose of our samples. Therefore, in theory, sperm DNA damage after only 9 mo in space will not be that obvious. However, in this study, although the space sperm samples did not show changes in morphology or decreased fertilization rates after ICSI, the DNA damage in the space sperm samples, as determined by the comet assay and gamma-H2AX immunostaining, was slightly increased compared with the ground control samples, irrespective of mouse strain. Previous space studies have shown that frozen human lymphoblastoid cells launched and preserved for 134 d in a freezer on the ISS sustained more damage to nuclei than the theoretical damage from the predicted dosage of radiation (28, 29); therefore, it is likely that the DNA damage caused by space radiation accumulated in the sperm nuclei until they were returned to Earth. Another possible explanation lies in the difference between artificial radiation in ground-based studies and actual space radiation. When fruit flies were launched into space on the Space Shuttle, although the flight duration was only 8 d, the frequencies of sex-linked recessive lethal mutations were 2- to 3-fold greater in these flies compared with the ground-based controls (40). That report suggested that the frequency of DNA damage will increase when biological samples are exposed to real space radiation. Taken together, the foregoing findings suggest that when frozen or freeze-dried spermatozoa are preserved in space, DNA damage from space radiation will be greater than that in samples held on Earth, even over short periods.

Importantly, the birth rate of offspring from the space sperm samples did not decrease compared with that from ground control sperm samples, even with the apparent increase in DNA damage to the nuclei during space flight. The comet assay on single spermatozoa revealed DNA damage in many spermatozoa. In contrast, with gamma-H2AX immunostaining of individual male pronuclei derived from space sperm samples, although the mean level of DNA damage was slightly higher than that in controls, the difference was smaller than that seen with the comet assay, and there were no significant differences in any strain except the BCF1 strain. Why only the BCF1 strain was significantly different is not clear, but the difference was not great, and some of the male pronuclei in this study did not show any DNA damage. Thus, it appears that sperm DNA damage was decreased or repaired after fertilization.

Our whole genome analysis also showed that the offspring derived from space sperm samples were similar to the offspring derived from the ground control sperm samples. It is well known that oocytes and zygotes have a strong DNA repair capacity (25–27); therefore, it is likely that any DNA damage in the space-preserved sperm nuclei was repaired after fertilization and thus had no ultimate effect on the birth rate of offspring. If this is so, then the DNA repair capacity in the oocyte cytoplasm needs to be quantified to evaluate the possibility of applying ART or AI to human or domestic animals in space habitats using long-term cryopreserved spermatozoa. In current ground-based AI procedures, sperm samples are often cryopreserved for more than

10 y; however, we found increased DNA damage in spermatozoa after only 9 mo in space. If sperm samples are to be preserved for longer periods in space, then it is likely that DNA damage will increase and exceed the limit of the oocyte's capacity for repair. Testing this will require more extensive sperm preservation experiments in space. If the DNA damage occurring during long-term preservation is found to have a significant effect on offspring, we will need to develop methods to protect sperm samples against space radiation, such as with an ice shield, to enable future animal breeding in this environment.

In addition, sperm preservation in the event of disasters on Earth will be an important tool for maintaining the genetic diversity of mammalian species, much like plant seed preservation in the Svalbard Global Seed Vault. Although current sperm drying methods can maintain nuclear integrity for only a limited number of years at ambient temperature, this time eventually will be extended. Reliability likely will be obtained before the beginning of the "Space Age," given that preservation periods have already been extended from a few months (31) to 2 y (36) by improving the drying method. In addition, drying conditions might be better than traditional cryopreservation for long-term preservation, because tardigrades can survive in extreme environments, such as space, only in a dehydrated state of dormancy (41). Once the reliability and integrity of space-preserved spermatozoa can be demonstrated, underground storage on the Moon, such as in lava tubes (42), could be among the best places for prolonged or permanent sperm preservation because of their very low temperatures, protection from space radiation by thick bedrock layers, and complete isolation from any disasters on Earth.

Methods

Animals. C56BL/6N, BDF1 (C57BL/6 × DBA/2), BCF1 (C57BL/6 × C3H/He), and 129B6F1 male mice carrying the green fluorescent protein (GFP) gene (GFP-tg-129/Sv × GFP-tg-C57BL/6), aged 3 mo, were used to collect spermatozoa. The C57BL/6N, BDF1, and BCF1 mice were purchased from Shizuoka Laboratory Animal Center, and the 129B6F1 mice were bred in our mouse facility. The C57BL/6N and BDF1 mice, age 8–10 wk, were used to produce oocytes. The surrogate pseudopregnant females used as embryo transfer recipients were ICR strain mice mated with vasectomized males of the same strain. On the day of the experiments, or after completion of all experiments, the mice were euthanized by CO_2 inhalation or by cervical dislocation. All animal experiments were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation of the RIKEN Center for Developmental Biology and by the Institutional Committee of Laboratory Animal Experimentation of the University of Yamanashi.

Preparation of Freeze-Dried Spermatozoa. Both epididymides were collected from male mice, and the ducts were cut with sharp scissors. A few drops of the dense sperm mass were placed into a centrifuge tube with 2 mL of CZB medium (43), followed by incubation for 30 min at 37 °C in 5% CO_2. After sperm concentration measurements, 50-μL aliquots of the sperm suspension were divided into 30 glass ampules. These ampules were frozen using liquid nitrogen and then freeze-dried (EYELA FDU-2200; Tokyo Rikakikai and FreeZone; Labconco). The cock of the freeze-drying machine was opened for at least 3 h until the samples were completely dry. After drying, the ampules were sealed by melting the necks using a gas burner under vacuum conditions, then kept in a –30 °C freezer until use.

Sperm samples were prepared from 70 male mice of four different strains. The quality of each lot was checked by examining its fertility (see below) using one or two ampules from each mouse. From these results, we selected samples from 12 mice: three from the C57BL/6 strain, four from the BDF1 strain, three from the BCF1 strain, and two from the 129B6F1 strain expressing GFP. These ampules and their data are referred to as the "original sperm" samples. Each ampule was wrapped in polyimide film and then the ampules were further wrapped in groups of four (Fig. 1 *A* and *B*). All ampules from 12 male mice (total of 48 ampules) were placed into small cases for space preservation (Fig. 1*C*) or as a ground control.

Launch to the ISS and Return to Earth. The sample cases, including the Bio PADLES radiation monitors (Fig. 1*C*), were launched aboard the H-II Transfer Vehicle KOUNOTORI 4 on August 4, 2013, at room temperature. After arrival at the ISS, astronauts stored them in a –95 °C freezer on August 10, 2013. Nine months later (on May 19, 2014), the sample case was retrieved from the freezer and then returned to Earth on the SpaceX-3 vehicle at ambient temperature. Therefore, these space sperm samples were exposed to cosmic radiation for 288 d. On May 21, 2014, the sample case was delivered to NASA and placed into a –80 °C freezer. This was then transported to our laboratory. These samples are designated the space sperm samples.

At the same times and for the same durations as the launching and storing of the sample case, a control sample case was exposed to room temperature, then frozen at –95 °C and stored in a freezer at the Tsukuba Space Center in Japan. These are designated the ground control sperm samples.

Space Radiation Dosimetry. Bio PADLES (TLD/CR39) monitoring devices (Fukuvi Chemical Industry) were used to measure radiation dosages. These include CR-39 PNTDs. The devices were placed inside the cases (Fig. 1*C*) of space and ground control sperm samples. After the sample cases were recovered, the analysis was initiated on June 2, 2014. Pictures of radiation-etched pits reflecting the tracks of atomic nuclei accumulated during space flight (Fig. 1*D*) were used to calculate the total radiation dose (44).

Analysis and Scoring of Comet Slides. The single-cell gel electrophoresis technique (i.e., the comet assay) measures DNA damage, including double- and single-strand breaks (45). Here comet assays to detect sperm DNA damage were performed according to the manufacturer's protocol (Trevigen). In brief, sperm specimens were collected from ampules immediately after opening and then rehydrated in water. Space and ground control sperm samples derived from the same male mouse were mounted on six slides, and 100–300 sperm heads were analyzed following electrophoresis. In some cases, spermatozoa had aggregated and were difficult to measure. To standardize the results among the different mouse strains, the length of each DNA comet "tail" was divided by the mean length of the ground control results in each strain.

Immunostaining of Zygotes. In histone H2AX, an H2A variants, the serine at position 139 is rapidly phosphorylated within minutes after DNA damage. The phosphorylated form of H2AX, designated gamma-H2AX, forms foci at the sites of DNA damage, which then serve as platforms to recruit various repair and cell cycle checkpoint proteins (46). Therefore, gamma-H2AX foci formation was used as a marker of DNA double-strand breaks in male and female pronuclei, and histone H3K9me2 signals were used to distinguish female pronuclei. The primary antibodies used were an anti–phospho-H2AX (Ser139) rabbit polyclonal antibody (1:500; Millipore Merck) and an anti-histone H3 (dimethyl K9) mouse monoclonal antibody (1:500; Abcam). The secondary antibodies used were Alexa Fluor 488-labeled goat anti-mouse IgG (1:500; Molecular Probes) and Alexa Fluor 568-labeled goat anti-rabbit IgG (1:500 dilution; Molecular Probes). DNA was stained with DAPI (2 μg/mL; Molecular Probes). The brightness of whole male pronuclei was measured using ImageJ and then subtracted from the brightness of the zygote cytoplasm.

ICSI and Embryo Transfer. To generate offspring from preserved spermatozoa, oocytes were collected from C57BL/6N or BDF1 female mice. Most experiments used BDF1 oocytes irrespective of the male mouse strain. Only when next-generation sequencing was performed for genomic analysis were C57BL/6 oocytes were used for C57BL/6 male mouse ICSI to generate pure inbred offspring. When ampules were opened, 50 μL of water was immediately added to rehydrate the sperm samples, and ICSI was initiated using four or five micromanipulators as described previously (47).

At 0.5 d postcoitum (dpc), embryos were transferred into the oviducts of pseudopregnant ICR strain female mice that had been mated with a vasectomized male the night before transfer. At 18.5–19.5 dpc, the offspring were delivered by caesarean section. Some embryos were cultured in 5% CO_2 in air for 5 d in CZB medium at 37 °C to examine their in vitro developmental potential and the quality of blastocysts.

Immunostaining of Blastocysts. To evaluate the quality of blastocysts derived from space sperm samples, immunofluorescence staining of blastocysts was performed as described previously (48). The primary antibodies used were an anti-CDX2 mouse monoclonal antibody (1:200; BioGenex) to detect TE cells and an anti-Oct3/4 rabbit polyclonal antibody (1:500; MBL) to detect ICM cells. The secondary antibodies used were Alexa Fluor 488-labeled goat anti-mouse IgG (1:500; Molecular Probes) and Alexa Fluor 564-labeled goat anti-rabbit IgG (1:500; Molecular Probes). DNA was stained with DAPI (2 μg/mL; Molecular Probes).

RNA Isolation and RNA Sequencing. In this study, both space and ground control sperm samples were obtained from the same male C57BL/6N mouse

strain, and fresh oocytes were collected from C57BL/6N female mice. Therefore, the offspring were of a pure C57BL/6N strain. At 18.5 dpc, fetuses were collected by cesarean section. Several organs from seven pups (four females, neonate IDs S2, S3, S4, S7, and three males, neonate IDs S1, S3, S6) from space sperm samples and six pups (one female, neonate ID G1; and five males, neonate IDs G2–G5) from ground control sperm samples were collected, immediately frozen in liquid nitrogen, and kept at –30 °C until use. Brain tissues were used for further analysis. Total RNA was purified using the AllPrep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's instructions. The library for RNA sequencing (RNA-seq) studies was prepared using the Kapa Stranded RNA/mRNA-Seq Kit (Illumina). The RNA-seq library was sequenced for 36-base single-end RNAs using an Illumina GAIIx machine with a TruSeq SBS kit v5–GA kit (Illumina). The resulting sequence data were mapped against the mouse reference genome sequence (GRCm38/mm10) using bowtie2 (49), followed by calculation of the reads per kilobase of exon per million mapped reads (RPKM) value for each gene using the Bioconductor package DEGseq (50). Clustering analysis and principal component analysis were performed with Cluster 3.0 (51).

Statistical Analysis. The comet DNA breakage assay results were evaluated using the Wilcoxon–Mann–Whitney nonparametric test, the gamma-H2AX assay results were evaluated using the Student *t* test, and the birth rate was evaluated using the χ^2 test. RNA-seq analyses were evaluated using the Wilcoxon–Mann–Whitney nonparametric test adjusted for a false discovery rate, and differentially expressed genes between space and control pups were statistically analyzed using the DESeq2 package (52) with the raw sequence reads count. $P < 0.05$ (birth rate and gamma-H2AX staining) or $P < 0.01$ (others) was considered statistically significant.

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