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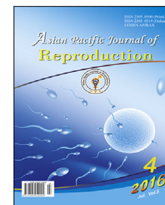

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Syngenic grafting of a whole juvenile male gonadal tissue into the adult testes confers successful spermatogenesis in mice

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ABSTRACT

Objective: To examine whether functional spermatozoa can be obtained when a whole male gonadal tissue (testes, epididymides, and fat) isolated from neonatal mice is grafted underneath adult mouse testes.

Methods: Neonatal (1-day-old) male gonadal tissue, isolated from enhanced green fluorescent protein (EGFP)-transgenic (Tg) mice (C57BL/6-Tg(ACTB-EGFP)1Osb/J), was inserted deep in the testis of a non-Tg recipient mouse through a tunica albuginea incision. Two months after transplantation, the fluorescent grafted tissues were retrieved from recipient mice.

Results: Histological analysis demonstrated that epididymal architecture was well developed and that spermatogenesis in the testis occurred in 30–60% of each seminiferous tubule of all the grafted tissues examined. Interestingly, motile spermatozoa could be successfully retrieved from the portion corresponding to the cauda epididymis in 1 of the 7 transplants obtained. These obtained spermatozoa had transgenes and could support embryonic development when intracytoplasmic sperm injection was performed using frozen-thawed spermatozoa.

Conclusion: This present technique will be useful for study in various biological fields including the rescue of Tg lines with lethal postnatal phenotypes and cloned animals that die immediately after birth.

1. Introduction

Spermatogenesis is a productive and highly organized process that generates a virtually unlimited numbers of sperm during adulthood. Moreover, recent advances have opened new avenues for the preservation of male gonadal function. For example, the advent of germ cell transplantation in mice [1,2], domestic animals [3,4], and primates [5,6], the *in vitro* culture of male germ cells [7,8], and the generation of immortalized cell lines from male germ cell lineages [9,10] have been developed to manipulate spermatogenesis, develop new strategies for the preservation of endangered species, and analysis of testicular defects.

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Subcutaneous grafting of small pieces of testis tissues from neonatal mouse [11,12], rabbit [13], cat [14], sheep [15], pig [15–20], bovine [21–23], horse [24], rhesus macaque [25–27] and human [28,29] into immunocompetent mouse hosts revealed that the gametogenic competence of the grafted testis is maintained and sometimes enhanced. Interestingly, sperm recovered from these testis grafts were viable and functional when intracytoplasmic sperm injection (ICSI) was performed on oocytes [12,13,16,19,25,30]. These results suggest that the growth and differentiation of testicular tissues can occur in the *in vivo* environment of a host mouse. We therefore tested whether a whole male gonad containing epididymides, dissected from a newborn animal, can survive and differentiate into mature tissues capable of generating functional sperm after transplantation into an appropriate place in an immunocompetent mouse.

In the present study, we grafted juvenile male gonadal tissues, dissected 1 d after birth, underneath the testicular capsule of a syngenic adult male testis, to examine whether the sperm in the gonadal graft could exhibit maturation of sperm with active motility. We used enhanced green fluorescent protein (EGFP)-

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transgenic (Tg) mice gonad donors and non-Tg syngenic mice as hosts. Two months after grafting, the grafts were dissected from the host testes and then subjected to several assays, including histological analysis to evaluate the structural integrity of the graft, and functional analysis to evaluate the developmental capacity of sperm recovered from the graft using assisted reproductive technology such as ICSI. Our findings support the notion that an ectopic location, such as the internal portion of a testis, can provide an adequate environment for juvenile male gonads to perform gametogenesis.

2. Materials and methods

2.1. Donor gonads

Whole donor male gonads containing epididymides (Figure 1a) were dissected from neonatal C_{57}BL/6 male Tg pups [heterozygous (Tg/+) for the transgene; derived from C_{57}BL/6-Tg(ACTB-EGFP)1Osb/J line] that express EGFP systemically [31] 1 d after birth. The Tg pups were confirmed by inspection of the surface of their body for expression of EGFP-derived fluorescence under a fluorescence stereomicroscope, as described below. The excised gonads were kept in ice-cold Dulbecco's modified phosphate-buffered saline with Ca^{2+} and Mg^{2+} (DM-PBS) (Invitrogen Co., Carlsbad, CA), for no more than 1.5 h, prior to grafting. In addition, some gonads were fixed in cooled DM-PBS containing 4% paraformaldehyde (Nacalai Tesque, Inc., Kyoto, Japan) for 24–48 h to serve as a reference for neonatal gonadal development; histological processing was carried out as described below.

2.2. Experimental surgery

Eight-to 15-week-old non-Tg (+/+) males derived from C57BL/6-Tg(ACTB-EGFP)1Osb/J line were used as graft recipients. For grafting, mice were anesthetized and one skin incision 4–5 mm was made on the dorsal side near the penis. Testes were pulled out and exposed on the dorsal surface (Figure 1b). A small slit was made on the testicular capsule by fine-tip dissection scissors (Napox R-12; Natsume Seisakusho Co., Tokyo, Japan) (arrow in Figure 1c). The whole male gonadal tissue excised from the Tg neonate was then inserted beneath the testicular capsule of the host using watchmaker's #5 forceps (Natsume Seisakusho Co.) (Figure 1d). Immediately after surgery, a portion of the graft is often expelled and appears to be exposed outside the testis (arrow in Figure 1e). However, this does not mean that grafting failed, as the graft grew well as shown in Figure 2a and b. Both of the recipient's testes obtained grafts, after which they were then returned to their original position within the scrotum. Recipient mice were fed *ad libitum* for 2 months prior to inspection. All animal experiments were approved by and performed under the guidance of the Animal Care and Use Committee at the University of Juntendo.

2.3. Analysis of grafts after surgery

Two months after surgery, grafts were dissected from the testis and inspected for expression of EGFP-derived green fluorescence under a fluorescence stereomicroscope, as shown schematically in Figure 2c. The dissected grafts were generally “oval” shaped, as shown in the right of Figure 2b. Grafts were classified as “well developed” if the size was >5 mm in length and > 2 mm in width. If the size of the graft was < 5 mm in length and < 2 mm in width, it was judged to be “poorly developed”, and were subjected to histological analysis without further dissection, as described below. Grafts judged as “well developed” were subjected to isolation of spermatozoa, as described in Figure 2c. The portion that appeared to correspond to the cauda epididymis was minced by fine-tip dissection scissors in a drop (~500 μL) of TYH medium [32] in a 35-mm

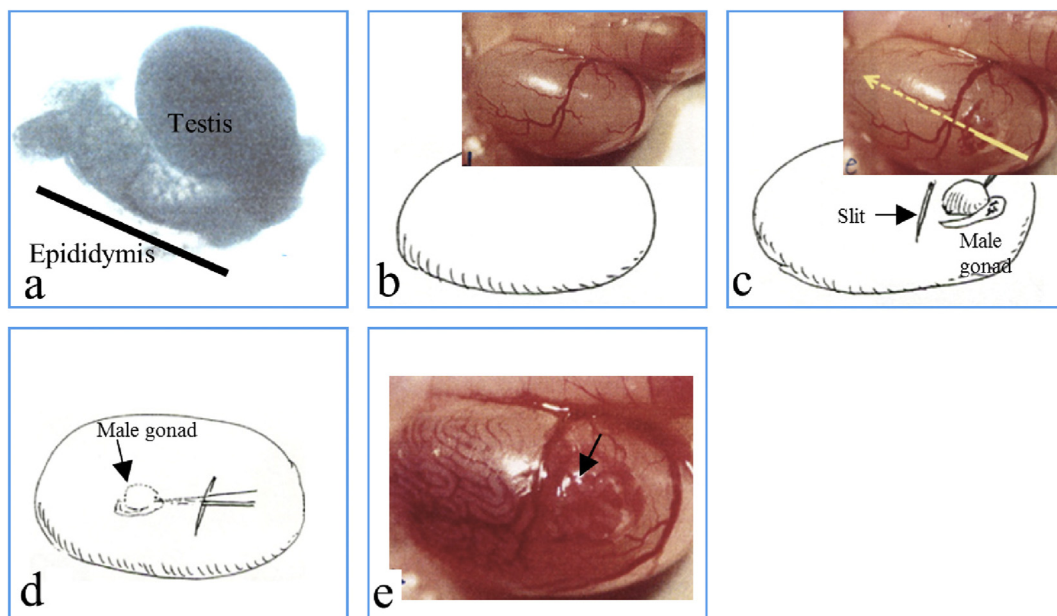


Figure 1. Procedure for intratesticular grafting of a whole juvenile male Tg gonads underneath the testicular capsule of a syngenic non-Tg host.
At first, a whole juvenile male Tg gonads are dissected (a). Next, a testis is pulled out and exposed on the dorsal surface skin of an adult non-Tg syngenic host mouse (b). A small slit is made on the testicular capsule by fine-tip dissection scissors. The excised whole juvenile male gonad is then inserted beneath the testicular capsule of the host mouse along the dotted arrow shown in the inset (c, d). Immediately after surgery, a portion of the graft is often expelled and appears to be exposed outside the testis (arrow in e). However, this does not mean that the graft failed since the graft subsequently grows well. The treated testis is then gently returned to the original position within the scrotum.

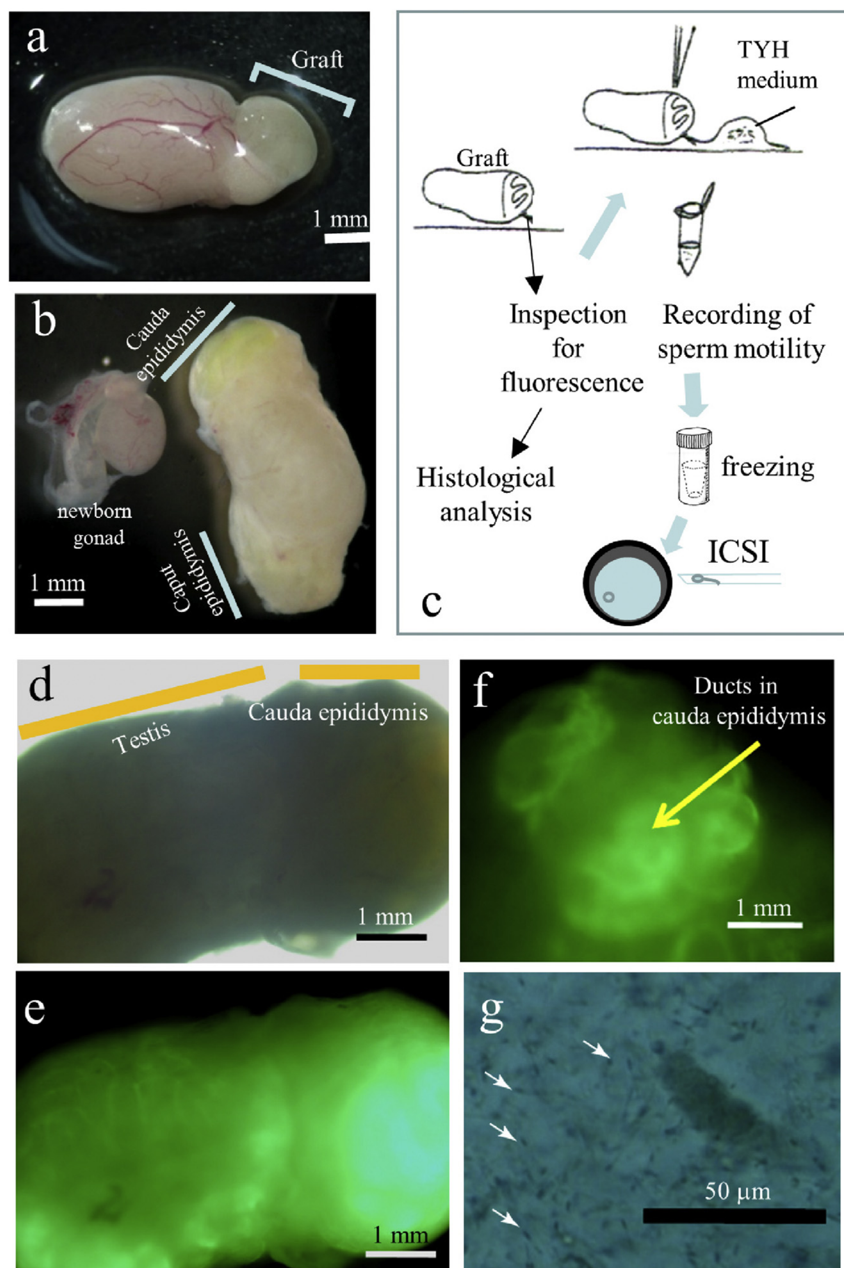


Figure 2. Isolation of grafts for checking sperm formation.
a, b, The grafts 2 months after surgery. Note that the graft is easily discernible from the host testis (a), since it often protrudes outside of the testis. The size of the graft (right in b) appears to be 3–5 folds larger than that of the neonatal male gonadal tissue (1 d after birth) (left in b). c, Schematic representation of the analytical process of graft isolation. d, e, Observation for EGFP-derived fluorescence under light (d) and UV illumination (e). f, The portion at the edge of the graft that appears to correspond to the cauda epididymis, exhibits the brightest ductal structure (indicated by arrow), which is evident under UV illumination. g, Microphotograph of isolated motile spermatozoa (arrows) with cellular debris from the graft #1-1.

suspension culture dish (Thermo Scientific/Nunc; Thermo Fisher Scientific, NY), which had been covered with paraffin oil (Nacalai Tesque, Inc.) to block evaporation, and then left for over 1.5 h at room temperature. During this period, sperm motility was checked under a light microscope and photographed. In addition, the number of sperm was calculated using a disposable hemocytometer (C-Chip; NanoEnTek Inc., Seoul, Korea). After observation, an aliquot (100 µL) was subjected to isolation of genomic DNA for PCR-based identification of a transgene, and the other remaining sperm suspension was pelleted by brief centrifugation and then cryopreserved in 18% raffinose and 1.75% glycerol in M2 medium (freezing solution), as described by Tada *et al.* [33] with modifications. Briefly, 50 µL of freezing solution containing spermatozoa was transferred to a cryotube (Nunc, Roskilde, Denmark) and then kept in a freezing container (BICELL; Nihon Freezer Co., Ltd., Tokyo, Japan) for 24 h at −80 °C. After cooling, the cryotube was preserved in a −196 °C storage tank until ICSI. Thawing of frozen spermatozoa was performed by keeping the cryotube in 40 °C water for 2 min followed by suspension of spermatozoa in HEPES-mCZB [34,35].

Grafts judged as “well developed” were fixed as described in the “Donor gonads” section. The fixed samples were then transferred to 70% (v/v) ethanol and stored at room temperature prior to paraffin embedding and subsequent sectioning at a thickness of 5 µm. Tissue sections were stained with hematoxylin and eosin.

The status of testicular maturation was judged by seminiferous tubule (ST) lumen formation (LF) and spermatogenesis within STs. A simple quantitative approach was used by determining the percentage of STs with mature spermatozoa within a graft.

2.4. Oocyte collection, intracytoplasmic sperm injection (ICSI), culture and embryo transfer

Oocytes were collected from C57BL/6J female mice superovulated with 10 IU of pregnant mare's serum gonadotropin (PMSG) (Peamex; Sankyo Lifetech Inc., Tokyo, Japan) followed by 10 IU of human chorionic gonadotropin (hCG) (Sigma–Aldrich; Missouri) approximately 48 h later. Females were sacrificed 14 h after hCG injection for collection of metaphase II (M II) oocytes. The complex of oocytes and cumulus cells were released from oviducts into 0.1% hyaluronidase (Sigma–Aldrich) in HEPES-mCZB and then kept for 2 min at 35 °C to disperse cumulus cells. The cumulus-free oocytes were washed and maintained in modified Whitten's medium (mW; [36]) at 37 °C in an atmosphere of 5% CO_2 in air until ICSI.

ICSI was carried out according to the method developed by Kimura and Yanagimachi [37] with slight modifications. The ICSI dish (Falcon Petri Dish; Corning Inc., NY) contained a drop of HEPES-mCZB and HEPES-mCZB containing 12% polyvinylpyrrolidone (Mr 360 000; Sigma–Aldrich) needle-cleaning drop covered with paraffin oil. Injections were performed by micromanipulators (Leica, Wetzlar, Germany) with a PMM-150 FU piezo-impact drive unit (Prime Tech Inc., Ibaragi, Japan) using a blunt-ended mercury-containing injection pipette with an inner diameter of approximately 6 µm. A single frozen-thawed spermatozoon was drawn, tail first, into the injection pipette and moved back and forth until the head-midpiece junction was at the opening of the injection pipette. The head was separated from the midpiece by applying one or more piezo pulses. After discarding the midpiece and tail, the head was redrawn into the pipette and injected immediately into an oocyte. Sperm-injected oocytes were washed with mW and cultured in 20 µL drops of the same medium covered with paraffin oil at 37 °C in 5% CO_2 in air. The oocytes were examined approximately 6 h after ICSI for survival and activation. The oocytes with two well-developed pronuclei were recorded 7 h after ICSI to assess fertilization ability. Two-cell embryos, developed from oocytes fertilized by ICSI, were cultured for approximately 24 h and then transferred to the oviducts of recipient females.

Nine to twelve 2-cell embryos were transferred into each oviduct of pseudopregnant ICR recipient females (aged 10–18 weeks; Charles River Laboratories Japan, Inc., Kanagawa, Japan) that were mated with vasectomized ICR male mice (aged 15–24 weeks; Charles River Laboratories Japan, Inc.). The embryo transfer to the pseudopregnant females was performed on the day that the vaginal plug was detected.

2.5. PCR analysis

Genomic DNA was extracted by adding 200 µL of lysis buffer [comprising of 0.125 µg/mL of proteinase K, 0.125 µg/mL of Pronase E, 0.32 mol/L sucrose, 10 mmol/L Tris–HCl (pH 7.5), 5 mmol/L $MgCl_2$ and 1% (v/v) Triton X-100] onto the sperm-containing pellet and incubating for 2–3 d at 37 °C, and subsequent extraction with phenol/chloroform. The supernatant was next ethanol-precipitated with the aid of GenTLE® Precipitation Carrier (#9094; Takara Bio, Inc., Ohtsu, Japan). The precipitated DNA was then dissolved in 20 µL of sterile water. The DNA was stored at 4 °C.

The DNA (~10 ng) was subjected to PCR [for identification of cytomegalovirus enhancer (CMVE) sequence in the transgenes in C57BL/6-Tg(ACTB-EGFP)1Osb/J line] in a volume of 20 µL, using the PCR conditions previously described [38]. The primer set (5′-GGG TCA TTA GTT CAT AGC C-3′ and 5′-GGC ATA TGA TAC ACT TGA T-3′) is expected to yield 215-bp PCR products. PCR conditions were as follows: *Taq* polymerase (Promega, Madison, WI), 1 min at 94 °C, 30 s at 94 °C, 30 s at 60 °C, and 20 s at 72 °C for 35 cycles, followed by a final extension step of 7 min at 72 °C. As a positive control, CMVE-containing plasmid DNA (~5 ng; derived from pCE-29 [38]) was concomitantly PCR-amplified. Water was used as a negative control.

2.6. Observation of fluorescence

Samples were examined under a fluorescence microscope (LEICA DMI 3000 B; Leica Microsystems GmbH, Wetzlar, Germany) for detection of EGFP-derived green fluorescence. Micrographs were taken using a digital camera (FUJIX HC-300/OL; Fuji Film, Tokyo, Japan) attached to the fluorescence microscope and images were printed using a Mitsubishi digital color printer (CP700DSA; Mitsubishi, Tokyo, Japan).

2.7. Statistical analysis

The rates of 2-cell embryo and blastocyst formation were scored and evaluated. The percentage data were arcsine transformed and subjected to analysis of variance (ANOVA) using the StatView program (Statistical Analysis System, SAS Institute, Cary, NC). P-values < 0.05 were considered statistically significant.

3. Results

3.1. Graft recovery

The growth of the grafted tissues was easily observed since they existed as protrusions from the host testis (Figure 2a). At the time of death, the grafts were photographed to document the survival and growth of the grafts. Table 1 summarizes the data on the grafts recovered 2 months after surgery. All of the grafts survived and were healthy. Of a total of 7 grafts, 5 (71%) grew well, were oval shaped, and were 5–7 mm in length and 2–3 mm in width (as exemplified by the graft in the right of Figure 2b). These grafts had increased 2–4 fold in size since they were grafted (newborn gonad *vs*. the graft in the right of Figure 2b). These isolated grafts were strongly fluorescent under UV illumination (Figure 2d and e). Particularly, the edge of the graft contained ductal structure, which is a property of epididymal ducts and was clearly visible under a fluorescence stereomicroscope (arrow in Figure 2f). We suspected that the ductal structure at the edge of the graft corresponded to the cauda epididymis where active motile spermatozoa are stored. Among the grafts dissected, the graft numbered #1-1 (Table 1) exhibited a well-developed structure in the portion corresponding to the cauda epididymis. Therefore, we decided to obtain spermatozoa within the ductal structure by teasing the edge portion of the graft under TYH medium by watchmaker's #5 forceps, as schematically illustrated in Figure 2c. As expected, the released spermatozoa were actively motile like the isolated cauda

Table 1

Summary of intratesticular grafting of juvenile male gonads.

Name of grafts	Growth of grafts[a]	Treatment	No. seminiferous tubules with sperm/no. tubules examined (%)
1-1	Well-developed	Check for sperm mobility Frozen sperm for ICSI[b] Check for transgenes	NT
1-2	Poorly developed	Frozen[c]	NT
2-1	Well-developed	H–E staining[d]	7/23 (30.4)
2-2	Well-developed	H–E staining[d]	4/8 (50.0)
3-1	Well-developed	H–E staining[d]	39/65 (60.0)
3-2	Poorly developed	Frozen[c]	NT
4-1	Well-developed	H–E staining[d]	24/50 (48.0)

[a] Each graft was classified as "Well-developed graft" and "Poorly developed grafts", as shown in Materials and methods. [b] After delivery of sperm from the graft by teasing, spermatozoa were checked for their motility and then frozen for ICSI. [c] The samples were not subjected to sperm isolation, because the size was too small. They were immediately frozen for future histological analysis. [d] Sections were subjected to hematoxylin and eosin (H–E) staining. NT, not tested.

epididymal spermatozoa (arrows in Figure 2g). The concentration of sperm ranged from 1×10^5 to 5×10^5/mL, which was comparable to that of normal C57BL/6 epididymal sperm (not shown). These spermatozoa were then subjected to freezing to test their ability to fertilize oocytes and to support fetal development to full-term.

3.2. Generation of progeny

We next examined the developmental potential of frozen spermatozoa recovered from the graft #1-1. The cryopreserved spermatozoa were thawed and subjected to ICSI. Sperm isolated from the intact (control) cauda epididymis of C57BL/6N mice were also used as a control. After ICSI, the treated oocytes were cultured *in vitro* to the blastocyst stage. The results are shown in the upper panel of Table 2. Both groups produced 2-cell embryos with similar rates (85.0% *vs.* 93.3%; $P > 0.05$). However, the percentage of blastocyst formation in the experimental group was 9.8%, which was significantly lower ($P < 0.01$) than that (75.0%) in the control group. Notably, some developing blastocysts in the experimental group exhibited normal morphology (as shown by the arrow in Figure 3A).

To assess *in vivo* developmental potential of 2-cell embryos that are developing from oocytes fertilized with the graft #1-1-derived sperm, we transferred them to the pseudopregnant recipient females. The results are shown in the lower panel of Table 2. Of 44 embryos transferred to 3 recipients, 6 (13.6%) successfully produced offspring. Among 6 pups born, 3 exhibited fluorescence over their skin (arrows in Figure 3B). Furthermore, these born pups survived to maturity and showed normal fertility through mating experiments (data not shown).

We also performed PCR analysis to confirm that the spermatozoa retrieved from the graft can possess transgenes that have been integrated into chromosomes of donor Tg grafts. As shown in Figure 3C, the PCR-amplified 215-bp band, was in fact detected in the spermatozoa isolated from the graft #1-1.

Table 2

In vitro and *in vivo* developmental rate of oocytes fertilized with frozen-thawed sperm by ICSI.

Source of spermatozoa		No. surviving oocytes/no. oocytes used for ICSI (%)	No. 2-cell embryos/no. surviving oocytes (%)	No. blastocysts/no. 2-cell embryos (%)
In vitro	Graft#1-1	73/82 (89.0)	51/73 (85.0)	5/51 (9.8)
	Control (intact cauda epididymal sperm)	30/38 (78.9)	28/30 (93.3)	21/28 (75.0)
In vivo	Graft#1-1	76/85 (89.5)	44/76 (57.9)	6/44 (13.6) (Tg 3; non-Tg 3)
	Control (intact cauda epididymal sperm)	68/100 (68.0)	60/68 (88.2)	32/60 (53.3)

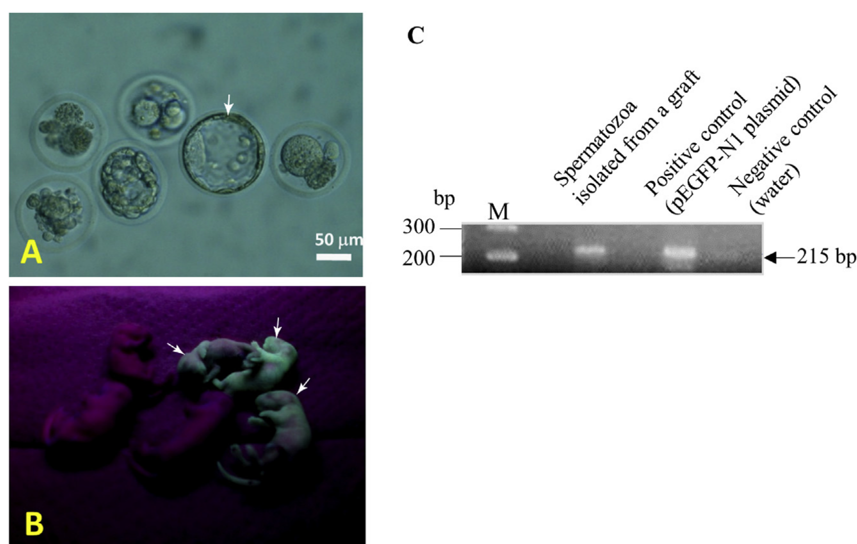
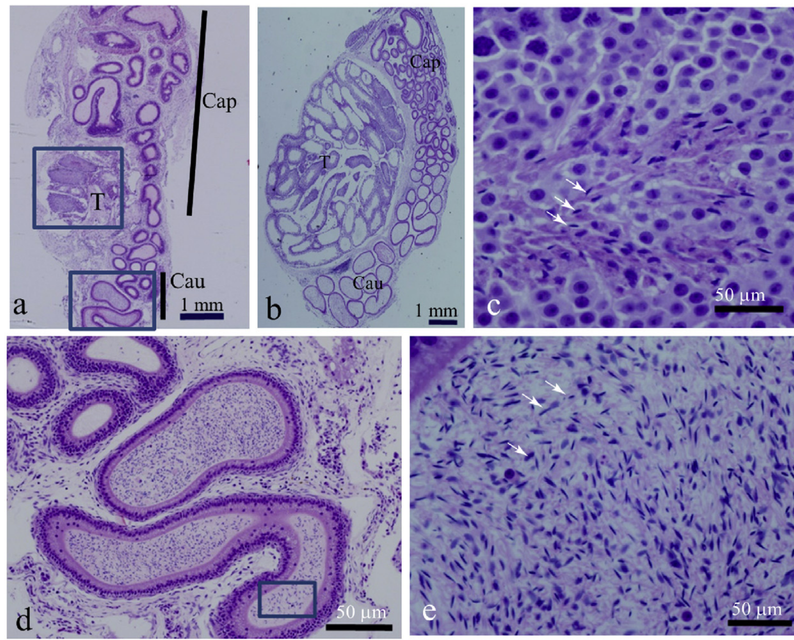


Figure 3. Analysis of offspring from the graft-derived sperm.

A, Blastocysts developed from ICSI with the graft-derived sperm. Note that some blastocysts (as exemplified by an embryo indicated by an arrow) exhibited normal morphology. B, Newborns developed from ICSI with the graft-derived sperm. Note that several pups (arrowed) exhibited fluorescence under UV illumination C, PCR analysis of sperm DNA isolated from the graft #1-1. M, 100-bp ladder markers.



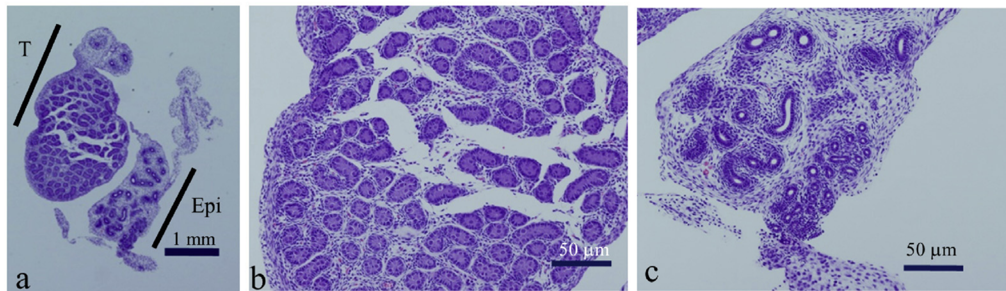


Figure 4. Histological analysis of grafts.
A, Histological sections of the grafts recovered 2 months after transplantation. a, b, Structure of 2 grafts (#2-2 and #4-1; see Table 1), all of which have well-developed STs in the testis (T), together with epididymal tissues including the caput epididymis (Cap) and the cauda epididymis (Cau). The region enclosed by boxes is shown as magnified figures in c-d. c, STs in the graft magnified from the T in the boxed region in a. Note the presence of mature sperm (arrows) in the lumen of the STs. d, The cauda epididymal portion in the graft magnified from the Cau in the boxed region shown in a. e, The cauda epididymal portion in the box of d further magnified. Note the presence of mature spermatozoa (arrows) within the lumen of the epididymal ducts. B, Histological section of a male neonate gonadal tissue (a) with testis (T) (b) and the epydidmal portion (Epi) (c). Note that the tubular cords of a testis are filled with gonocytes.

3.3. Spermatogenesis and ST development in the grafts

The most remarkable observation in the recovered grafts was the development of epididymal structures (corresponding to the caput epididymis, corpus luteum, and cauda epididymis), where mature spermatozoa are thought to be abundantly present. In subfigures a and b of Figure 4A, two representative histological sections are shown. Histological analysis of these recovered grafts revealed that the portion corresponding to the caput epididymis (shown by “Cap”) is located nearest to the testis and had several segments, characterized by the presence of ciliated epithelia lining the ductal lumen. Furthermore, the architecture that was localized most distant from the testis appeared to correspond to the cauda epididymis (shown by “Cau”), in which mature spermatozoa were found to be abundant (subfigure d of Figure 4A; arrows in subfigure e of Figure 4A). In the middle portion of the dissected grafts, a well-developed testicular structure (shown by “T” in subfigures a and b of Figure 4A) was always seen. Notably, 30–60% of the STs exhibited complete spermatogenesis, as evaluated by the presence of mature spermatozoa in the lumen of the STs (arrows in subfigure c of Figure 4A; Table 1). The other tubules lacked matured spermatozoa or were filled with liquid without any overt sperm (data not shown). These features found in the grafts recovered 2 months after transplantation are in contrast to those found in the juvenile gonadal tissues that comprise poorly differentiated cells such as seminiferous cords, Sertoli cells, and gonocytes (Figure 4B).

4. Discussion

In the past two decades, subcutaneous grafting of pieces of testicular tissues dissected from male neonates into immunodeficient mice (which is called “xenogenic grafting”) or normal mice (which is called “allogenic grafting”) has been employed [39]. In these cases, complete spermatogenesis is achieved in the grafts from mice, pigs, and rabbit, and is in some cases (e.g., pigs) enhanced [11]. However, production of motile spermatozoa has not yet been reported. This is solely due to the absence of epididymal tissues in the donor grafts upon transplantation. In general, acquisition of sperm motility occurs during transport of

testicular sperm to the epididymal ducts and the refractory period for forthcoming ejaculation. Since sperm recovered from the grafts are matured, but have no motility, the researchers have had to perform assisted fertilization such as ICSI to produce offspring [12,13,16,19,25,30]. In this context, our present system that depends on intratesticular grafting of a juvenile whole male gonadal tissue appears to be superior to previous approaches, because with the former system it was only possible to obtain mature spermatozoa with mortality. Unfortunately, in only one case could motile spermatozoa be successfully retrieved from the grafts. We then tried to obtain motile spermatozoa using the same method shown here but failed. Our major concern was how motile spermatozoa could be obtained from the grafts grown under a testicular environment. If our results can be replicated, it will be possible to obtain progeny via *in vitro* fertilization, which is simpler and more convenient than the ICSI-mediated production of progeny.

The location of grafting appears to be one of the critical factors that allow proper growth and differentiation of transplanted gonadal tissues. Most previous research has employed subcutaneous implantation such as grafting under the back skin of immunodeficient mice [12,16,21,22,25]. For example, a small piece of testicular tissues (ranging 1–2 mm in length; [11]) has often been used for grafting. In this case, inhibitory effects on the growth of the graft appear to be a few even though there is a continuous tight constraint between the skin and body muscle, probably due to the small size of the graft. However, the growth of larger grafts such as a whole neonatal male gonadal tissue including epididymides is likely amenable to this constraint. In contrast, grafting beneath the testicular capsule allows larger samples to grow. If protrusion of a part of the graft from a testis arises, it may grow normally since there is sufficient space that allows the graft to grow within the scrotum. Furthermore, the grafted testis returning to the inside of the scrotum also appears to be important, because the scrotum provides a suitable environment for a testis to grow properly under relatively lower temperatures compared to body temperature. Unfortunately, there are a few reports on intratesticular grafting. Toyooka *et al*. demonstrated that intratesticular grafting of *in vitro* generated germ cell precursor aggregates resulted in induction of full spermatogenesis from the grafts [40]. Van Saen *et al*. demonstrated that when a testicular tissue piece (1.5 mm × 1.5 mm × 1.5 mm) of a 5- to 7-day-old mouse was grafted through a fine incision in the tunica albugina, the grafts exhibited complete spermatogenesis [41].

In conclusion, ectopic grafting of a whole neonatal male gonad leads to induction of complete spermatogenesis along with the formation of epididymal ducts. In those epididymal tissues, active motile spermatozoa capable of supporting full-term fetal development were generated. Therefore, grafting of whole neonatal gonads including the epididymis can be used to preserve the fertility of animals that have died before reaching puberty.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgment

This study was partly supported by a grant (no. 23510299) from the Ministry of Education, Science, Sports, and Culture, Japan. NT and MS designed the study, analyzed the data, and drafted the manuscript. NT, FK, and EN performed grafting of testes, intracytoplasmic sperm injection and embryo transfer. EN was also involved in the molecular analyzes of PCR-amplified products. EI and IS critically revised the manuscript.

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