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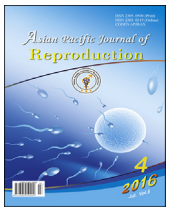

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Syngenic grafting of a whole juvenile male gonadal tissue into the adult testes confers successful spermatogenesis in mice

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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.

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

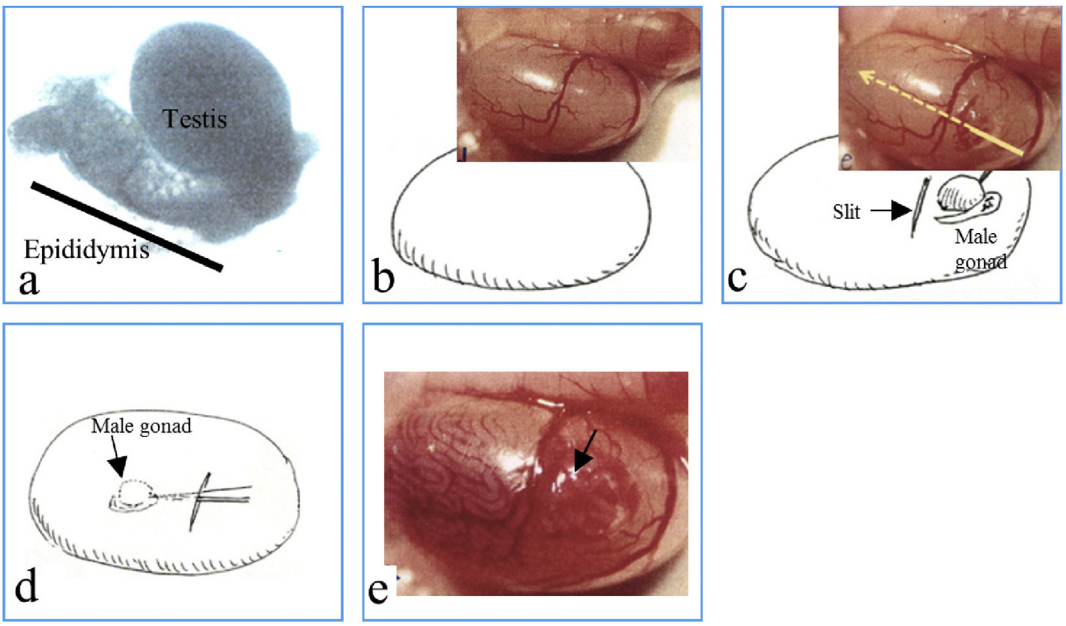

Figure 1. Procedure for intratesticular grafting of a whole juvenile male Tg gonads underneath the testicular capsule of a syngenic non-Tg host.
At first, a whole juvenile male Tg gonads are dissected (a). Next, a testis is pulled out and exposed on the dorsal surface skin of an adult non-Tg syngenic 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.