Sato, Clon Transgen 2012, 1:1
DOI: 10.4172/2168-9849.1000102

Cloning & Transgenesis
ISSN: 2168-9849

Research Article Open Access

# Functional Recovery of a Whole Ovary Transplanted Into Syngenic Testis in Mice

**Masahiro Sato[1]*, Masato Ohtsuka[2], Shingo Nakamura[3], Takayuki Sakurai[4], Satoshi Watanabe[5] and Yukiko Yasuoka[6]**

[1]*Section of Gene Expression Regulation, Frontier Science Research Center, Kagoshima University, Kagoshima, Japan*
[2]*Division of Basic Molecular Science and Molecular Medicine, School of Medicine, Tokai University, Kanagawa, Japan*
[3]*Department of Surgery, National Defense Medical College, Saitama, Japan*
[4]*Department of Organ Regeneration, Graduate School of Medicine, Shinshu University, Nagano, Japan*
[5]*Animal Genome Research Unit, Division of Animal Science, National Institute of Agrobiological Sciences, Ibaraki, Japan*
[6]*Department of Physiology, Kitasato University, School of Medicine, Kanagawa, Japan*

## Abstract

We previously demonstrated that a whole juvenile (2-week-old) mouse ovary transplanted into a syngenic host testis survived and exhibited normal folliculogenesis. The aim of this study was to test whether the graft possessed normal abilities such as ovulation. Whole ovaries were excised from a female transgenic mouse (2-week-old) ubiquitously expressing enhanced green fluorescence protein. The isolated ovary was then transplanted into the testis of a non-transgenic male (2-week-old), in which approximately 80% of the seminiferous tubules had been removed to create enough space for the graft to grow properly. Two months after transplantation, the ovary grafts were isolated from the testis and subjected to histological analysis. Some grafts were subsequently subjected to ovary transplantation to assess the capability of the grafted ovaries to ovulate oocytes. Two months after ovary transplantation, 3 of 8 ovaries grafted under the ovarian capsule successfully ovulated fluorescent oocytes in response to a surge of luteinizing hormone. These results indicate that intratesticular grafting of juvenile ovaries allows functional development of the graft. This system would be helpful for investigating oogenesis/folliculogenesis in ectopic environments as well as for rescuing ovaries with genetic defects.

**Keywords**: Ovary; Transplantation; Testis; EGFP; Tolliculogenesis; Seminiferous tubules; Ovulation

**Abbreviations:** Tg: Transgenic; STs: Seminiferous Tubules; EGFP: Enhanced Green Fluorescence Protein; PFA: Paraformaldehyde; LH: Luteinizing Hormone; FSH: Follicle-Stimulating Hormone; PBS (-): Phosphate-Buffered Saline without $Ca^{2+}$ and $Mg^{2+}$

## Introduction

Mammalian ovaries comprise follicles containing maturing oocytes, thecal cells, and interstitial cells. Control of folliculogenesis involves interactions between the pituitary gonadotrophins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), and intraovarian factors such as steroids, cytokines, and growth factors. The latter stage of folliculogenesis is strictly controlled by FSH and LH [1,2], both of which are preferentially produced in females. Therefore, it is possible that the development of mammalian ovaries placed in a male hormonal environment would be suppressed because of the presence of only small amounts of female-specific hormones and large quantities of male androgens. Earlier studies have demonstrated that the development of embryonic mouse ovaries is affected by transplantation into adult and embryonic testes [3]. According to Ozdenski et al. [3], the environment of adult testis causes severe restriction of ovarian growth and degeneration of oocytes, but fails to exhibit reversion of the differentiation of genetically female germ cells, a phenomenon called "mascularization." In addition, other groups have also observed this mascularization phenomenon [4-11]. This problem is, therefore, still a matter of discussion.

In contrast, intratesticular transplantation of a juvenile ovary (about 2 weeks after birth) into the environment of an adult testis does not appear to affect the quality of the graft itself. In our previous study in which young ovaries isolated from 2-week-old Enhanced Green Fluorescence Protein (EGFP)-expressing transgenic (Tg) females were transplanted into 4-week-old non-Tg male testes, no obvious adverse effects were noted in the ovarian grafts [12]. In these grafts, normal folliculogenesis in the grafts was recovered 2 months after grafting occurred. We think that the follicular growth of the 2-week-old mouse ovary is insensitive to the male hormonal environment, unlike in case of grafting embryonic ovaries.

Our main subject with regard to intratesticular grafting of juvenile ovaries is whether the grafts grown within a testis have the potential to ovulate oocytes. In this study, we have assessed this possibility by using an improved method for intratesticular grafting of juvenile ovaries.

## Materials and Methods

### Intratesticular grafting of an ovary

Intratesticular grafting of a juvenile mouse ovary was performed according to the method described by Sato et al. [12] with some modifications. Briefly, 2-week-old ovaries from homozygous female Tg mice of the MNCE-39 line, which systemically over expresses EGFP [13], were transplanted into 2-week-old non-Tg MNCE-39 male mice.

A small abdominal incision (approximately 1 cm in length) was made to expose the testes of recipient non-Tg males that were under pentobarbital-mediated anesthesia (Figure 1a). Approximately 80% of the seminiferous tubules (STs) were removed using suction with a 20-gauge needle attached to a 20-mL plastic syringe (Terumo, Tokyo, Japan), which created a space (Figure 1b and 1c) into which a single whole Tg ovary could be inserted with the aid of forceps

***Corresponding author:** Masahiro Sato, Section of Gene Expression Regulation, Frontier Science Research Center, Kagoshima University, Kagoshima, Japan, Tel: 81-99-285-3585; Fax: 81-99-285-3585; E-mail: masasato@ms.kagoshima-u.ac.jp

**Received** June 05, 2012; **Accepted** July 12, 2012; **Published** July 16, 2012

**Citation:** Sato M, Ohtsuka M, Nakamura S, Sakurai T, Watanabe S (2012) Functional Recovery of a Whole Ovary Transplanted Into Syngenic Testis in Mice. Clon Transgen 1:102. doi:10.4172/2168-9849.1000102

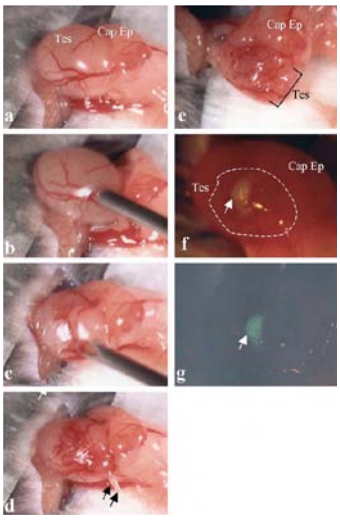


**Figure 1:** Procedure for intratesticular transplantation of an ovary from EGFP-expressing transgenic mice **(a)** Testes from a 2-week-old non-transgenic MNCE-39 mouse line were exposed following an abdominal incision. (**b-d**) Testis from which a portion of the seminiferous tubules was suctioned using a 20-gauge needle; remnant seminiferous tubules are visible outside the testis (indicated by arrows in **d**). (**e**) Testis after removal of about 80% of seminiferous tubules. (**f, g**) Ovary (arrows) from 2-week-old MNCE-39 transgenic mouse inserted into the testis immediately after the removal of the seminiferous tubules, showing bright EGFP fluorescence under UV + light illumination (arrow in **g**). In (**f**), the testis is indicated by dotted lines. Tes, testis; Cap Ep, caput epididymis.

(Figure 1d-g). After grafting, the hole in the testicular capsule was closed via treatment with instant glue (Aron Alpha; Konishi bond, Osaka, Japan), and the testis was then returned to its original place. The same surgical procedure was carried out for the other testis. Males were allowed to survive for 2 months, and then inspected for EGFP-derived fluorescence under a fluorescence microscope. Then, they were subjected to histological analysis and ovary transplantation.

Mice were maintained on a 12-h-light/12-h-dark schedule (lights on from 0700 to 1900 h), and were provided with food and water ad libitum. Experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals at the Tokai University*. All efforts were made to minimize the number of animals used as well as their degree of suffering before, during, and after surgery.

## Ovary transplantation

Grafting of an ovary underneath the ovarian capsule was performed according to the procedure described by Migishima et al. [14] with slight modifications. Recipient non-Tg females of the MNCE-39 line (8-10 week-old) were anesthetized with pentobarbital, and a small ventral incision (approximately 0.5 mm) was made to expose the ovary. The ovarian bursa was cut using microscissors (# MB-55; Natsume Seisakujo, Tokyo, Japan), and then, the bottom of an ovary was cut and removed with the same scissors (Upper panel of Figure 3A-a). The ovaries used for ovary transplantation were recovered from the testes that had been grafted with a juvenile Tg ovary. In case of the former ovary, the surface of the isolated ovary was trimmed by removing amorphous structures by using micro scissors. The trimmed ovary was then inserted under the ovarian bursa (Middle panel of Figure 3A-a). After grafting, the bursa was closed using Aron Alpha (Lower panel of Figure 3A-a). The same surgical procedure was carried out for the other ovary. Females were allowed to survive for 2 months, and then administered exogenous gonadotrophins. Upon induction of superovulation, they were first injected intraperitoneally (IP) with 5 IU of eCG (1700 h; ASKA Pharmaceutical, Tokyo, Japan), and 48 h later with 5 IU of hCG (1700 h; ASKA Pharmaceutical). Sixteen hours after hCG administration, the oviducts were dissected and inspected for fluorescence under a fluorescence microscope. Ovulated oocytes were isolated from the ampulla of these isolated oviducts and treated with 400 IU/mL hyaluronidase (Sigma Chemical Co., St. Louis, MO) to remove associated cumulus cells, as described previously [15].

## Inspection of fluorescence

For detection of EGFP fluorescence in the whole testis, the ovarian grafts isolated from a testis, the ampulla of an oviduct, and the isolated oocytes, we used an Olympus BX40 fluorescence stereomicroscope (Olympus, Tokyo, Japan) with DM505 filters (BP460-490 and BA510IF). For detection of EGFP fluorescence in the cryostat sections, an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan) with DM505 filters was used. Microphotographs were taken using a digital camera (FUJIX HG-300/OL; Fuji Film, Tokyo, Japan) attached to the fluorescence microscope, and printed out using a Mitsubishi digital color printer (CP700DSA; Mitsubinshi, Tokyo, Japan).

## Histological examination

Testes containing ovarian grafts were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline without $Ca^{2+}$ and $Mg^{2+}$, PBS (-), pH 7.2, at 4°C for 2 days. Each testis was then cut in half, and the inner surface was photographed under a BX40 stereomicroscope. These specimens were then dehydrated first in 0.25% sucrose in PBS (-) at 4°C for 4 days with gentle shaking, and then in 0.4% sucrose in PBS(-) at 4°C for 4 days. Next, the samples were embedded in OCT compound (Tissue-Tek [No. 4583]; Miles Scientific, Naperville, IL, USA) for cryostat sectioning. In some cases, sections were stained with hematoxylin and eosin, according to standard histological procedures.

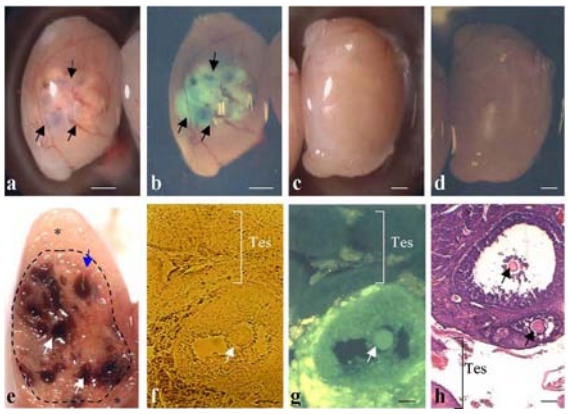


**Figure 2:** EGFP fluorescence and structure of ovarian grafts 2 months after intratesticular grafting (**a,b**) Grafted testes 2 months after the transplantation of a 2-week-old Tg ovary. The grafted ovary (arrows in **a**) photographed under light exhibits bright EGFP fluorescence under UV + light illumination (arrows in **b**); Bar = 1 mm. (**c, d**) An intact testis from a 2.5-month-old non-Tg mouse photographed under light (**c**) and UV + light illumination (**d**). The intact testis is negative for fluorescence. Bar = 1 mm. (**e**) The inner surface of the grafted testis (in which about 80% of the seminiferous tubules had been depleted) bisected after fixation in 4% paraformaldehyde. Note the presence of well-developed ovary (enclosed by dotted lines) with some atretic follicles. * indicates host seminiferous tubules. (**f, g**) Cryostat section of the sample under (**e**) light (**f**) and light + UV illumination (**g**); as shown in (**g**), there is no fluorescence in the host tissue (indicated by "Tes"), while green fluorescence is notable in the graft (containing follicular cells of an antral follicle and an oocyte (arrow in **g**). (**h**) Hematoxylin-eosin-stained section of a testis grafted with a juvenile ovary. There are well-developed antral follicles with an oocyte (arrow) and immature follicles. Tes, testis. Bar: 100 μm

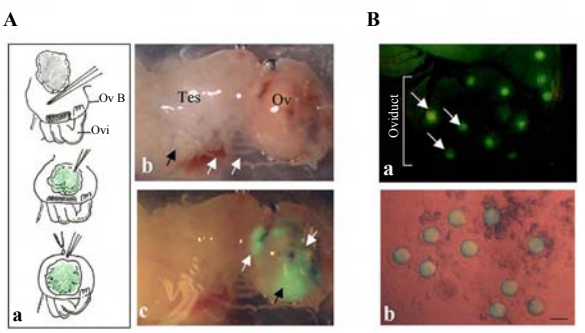


**Figure 3A:** (**a**) Schematic representation of the procedure for ovary transplantation. Prior to grafting the exogenous ovary, almost all portions of the endogenous ovary are removed after cutting the ovarian bursa (Ov B). The fluorescent ovary isolated from the grafted testis is then inserted underneath the ovarian bursa. Following this, the ovarian bursa is closed using Aron Alpha. Ovi, oviduct (**b, c**) Ovary (recovered from the grafted testis) photographed under light (**b**) exhibits bright EGFP fluorescence under UV + light illumination (arrows in **c**). Note that the seminiferous tubules (arrows in **b**) of the host non-Tg testis were often associated with the fluorescent ovarian graft, but they never fluoresced, as shown in (**c**). Tes, testis; Ov, ovary.

**Figure 3B:** (**a**) Ovulated fluorescent oocytes (arrows) present in ampulla 16 h after hCG administration exhibit bright EGFP fluorescence. Ovary and oviduct dissected from the recipient females were immediately observed for EGFP fluorescence by using a fluorescence microscope. (**b**) Fluorescent oocytes recovered from the ampulla shown in (**a**). Bar: 100 μm.

## Results

At 2 months post-surgery, almost all ovarian grafts (10/12 grafted per testis) survived, as evidenced by the bright green fluorescence beneath the testicular capsule (arrows in Figure 2a,b). The remaining 2 grafts fluoresced yellow throughout the entire body (data not shown), thus suggesting global death of the component cells. Testes carrying ovarian grafts appeared to be slightly smaller than normal testes (Figure 2c, d vs. a,b), probably because of the surgical reduction in the volume of STs. Two of these grafted testes were subjected to fixation, the fixed whole testes were then bisected, and the inner surface was then inspected under a light microscope. The ovarian grafts were clearly distinguishable by color from the host STs (area enclosed by dotted lines in Figure 2e). Notably, enlarged follicles filled with blood were frequently observed (arrows in Figure 2e), probably suggesting that they had been atretic. Examination of the cryostat sections of the testes revealed that the STs near the graft were completely negative for fluorescence ("Tes" in Figure 2f,g), suggesting the absence of extensive cell migration from the grafted ovaries into the testicular tissue. There were several well-developed fluorescent preantral and antral follicles containing normal oocytes (arrows in Figure 2f,g). Hematoxylin-eosin staining of the grafts also confirmed the above results (arrows in Figure 2h). These findings indicate that, at the least, some parts of the ovarian grafts within the host testis exhibited normal folliculogenesis.

The other remaining 8 ovarian grafts were subjected to ovarian transplantation to test whether the grafts isolated from the host testis could potentially ovulate oocytes. When the fluorescent ovarian grafts were isolated, some testicular components, including STs and interstitial cells, were tightly associated with the surface of the graft (arrows in Figure 3A-b). These residual cellular components were removed as soon as possible by microscissors prior to ovarian transplantation, which was performed by inserting the trimmed ovarian graft beneath the ovarian capsule of an ovary, from which almost all parts of the non-transgenic host ovary had been removed with the aid of microscissors, as depicted in Figure 3A-a. After placing the ovarian graft, the ovarian capsule was closed. Treated females were allowed to survive under normal conditions for 2 months, and then were subjected to hormonal treatment to induce superovulation. In the morning, 16 h after hCG administration, ovulated oocytes present at the ampulla of an oviduct were inspected under UV illumination. Some fluorescent oocytes were detected inside the oviduct (arrows in Figure 3B-a). When oocytes within an oviduct were delivered by flushing the oviduct, and then inspected for fluorescence, distinct fluorescence was observed in some oocytes (Figure 3B-b). Of a total of 4 female recipients subjected to surgery, 3 oviducts contained fluorescent oocytes (Table 1). Twenty-one of the 36 oocytes obtained were fluorescent. At this time, non-fluorescent oocytes were also obtained (Table 1), which may have been derived from the ovarian remnant of the recipient.

## Discussion

In this study, we improved the previously used method for intratesticular grafting of a juvenile ovary [12], and demonstrated that the grafted ovary still possesses the ability to ovulate oocytes when placed under a normal environment via ovary transplantation. Intratesticular grafting of an ovary was first performed by Sand [16,17], who found that the grafted ovary developed mature follicles, although the procedure remained unclear regarding the age of animal most suitable for obtaining donors and recipients and the most optimum stage of follicle maturation. Ozdzenski et al. [3] later grafted 11–13-day-old embryonic mouse ovaries into an adult testis and found that the grafts had severely restricted growth, but not mascularization. Takewaki [18] reported that most of the ovarian grafts survived for up to 2 months. We also observed normal folliculogenesis in the ovaries 2 months after intratesticular grafting of juvenile ovaries. These results suggest that the endogenous hormonal environment of males can support viable ovaries. Besides the testis, transplantation of intact or cryopreserved young or adult ovaries under the kidney capsule, into the abdominal wall, under the subcutaneous pocket, or into the uterine horn has been reported [19]. In almost all cases, follicular development of the grafted ovary occurred [19]. These results suggest that young, and possibly, adult, but not fetal, ovaries can grow and differentiate autonomously even in ectopic environments.

One of the major concerns about intratesticular grafting of an ovary is whether the graft has a potential to ovulate oocytes in response to exogenous gonadotrophins. The later stages of folliculogenesis (i.e., antral follicles) depend on the production of FSH and LH [1,2]. Since males do not produce as much FSH or LH as females, it remains

| Name of recipient mice[1] | Site of oviducts | Number and fluorescence of oocytes recovered 16 h after hCG administration |
|---|---|---|
| 1 | R<br>L | 0<br>12 (3, non-fluorescent; 9, fluorescent) |
| 2 | R<br>L | 2 (2, non-fluorescent)<br>6(4, non-fluorescent ; 2, fluorescent) |
| 3 | R<br>L | 0<br>0 |
| 4 | R<br>L | 6 (6, non-fluorescent)<br>10 (10, fluorescent) |

[1]A total of 4 female syngenic non-transgenic mice were used for ovary transplantation of the ovarian grafts recovered from testes, as described in Materials and Methods. Both host ovaries were replaced by the exogenous ovaries. Two months after ovary transplantation, females were subjected to hormonal treatment to superovulate oocytes. Sixteen hours after hCG administration, ovulated oocytes were isolated from the oviducts and examined for EGFP-derived fluorescence. R-right oviduct; L-left oviduct

**Table 1:** Summary of ovary transplantation of ovarian grafts recovered from testes.

unknown whether ovulation, the final stage of folliculogenesis, would have proceeded in ovarian grafts grown in the host testis without significant levels of FSH and LH. Therefore, we decided to retransplant the ovarian grafts under the female ovarian capsule wherein sufficient amounts of FSH and LH could be provided. Administration of exogenous gonadotrophins to females 2 months after ovarian transplantation, in fact, caused ovulation, although the frequency appeared to be relatively low (38%, 3/8 oviducts tested; Table 1). These results suggest that the ovary grafted and grown within a host testis does possess the ability to ovulate. In future, we will evaluate whether ovulated oocytes have the ability to fertilize with spermatozoa, and whether the fertilized oocytes can subsequently develop further.

Improvement of testicular grafting of an ovary appears to be one of the factors leading to improved recovery of function in the ovaries grafted within the host testis. In our previous experiments [12], only about 40%-50% of STs were removed, which created a limited space, thus allowing the grafted ovary to grow normally. In this study, we removed about 80% of STs by suction by using 20-gauge needle, which gave rise to a larger space wherein the grafted juvenile ovary could grow well (Figure 2e). Histological analysis of the grafted ovary revealed the presence of antral follicles with oocytes with a normal appearance (Figure 2f-h), suggesting that appropriate conditions were created using a method in which the graft grew without possible physiological pressure. This method would thus be useful for grafting in other tissues, in addition to the ovary. In conclusion, using a newly developed gene-engineering technique, we have found that ovarian grafts grow normally and exhibit normal folliculogenesis 2 months after surgical transfer into the host testis, and that grafted ovaries retain their function (ovulation upon hormonal induction) when re-implanted under the ovarian bursa of a female recipient. As mentioned earlier, whether ovulated oocytes recovered from ovarian grafts have the ability to be fertilized by epididymal mature spermatozoa is the next concern that we plan to resolve in future studies. Intratesticular transplantation of the ovary may be a unique method for evaluating the developmental potential of the ovaries in a condition wherein the levels of FSH and LH are significantly decreased, and may also be useful for rescuing ovaries with genetic defects.

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