Tracking the rejection and survival of mouse ovarian iso- and allografts in vivo with bioluminescent imaging

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Abstract

The applications of in vivo bioluminescent imaging (BLI) with a luciferase reporter gene occur widely across biomedical fields. Luciferase-transgenic mice are highly useful donors for tracking transplanted ovarian tissues. Realizing the full potential of this system may greatly benefit the study of the physiological behaviour and function of transplanted grafts, and the rapid and reliable evaluation of new transplantation protocols. The ovarian tissues of donor FVB/N-Tg(PolII–Luc)Ltc transgenic mice, with a luciferase transgene as the reporter, were transplanted into iso/allogeneic recipients. Rejection, ovarian function and BLI were quantitatively analysed in vivo over time. The BLI of the ovarian isografts revealed longer survival than that of allografts, even with cyclosporine A (CsA) treatment. The CD4⁺/CD8⁺ ratios of peripheral T-cells were significantly reduced in allografts compared with those in isografts (P < 0.0001) during rejection, whereas CD19⁺ cell numbers were higher in allografts. The infiltration of CD4⁺/CD8⁺ cells into the graft was unremarkable in isografts from day 1, but was strong in allografts from day 8 onwards. Hormone activity revealed complete oestrus cycles in the isografts but only the dioestrus stage in the allografts. These results demonstrate that BLI in vivo expedites the fast throughput and fate maps of ovarian grafts. The use of BLI to longitudinally monitor ovarian grafts for immunorejection demonstrated the short survival of allografts and the much longer survival of isografts. CsA treatment alone is ineffective against the acute rejection of ovarian allografts.


Introduction

New discoveries applied to the preservation of fertility have created an unprecedented need for assisted reproductive technologies in young patients undergoing cancer therapy or with premature ovarian failure (POF; Donnez et al. 2006). In the past decade, much effort has been devoted to oocyte and ovary cryopreservation for autotransplantation (Kim et al. 2001, Chen et al. 2003, Kuwayama et al. 2005).

A series of discordant monozygotic twins restored fertility after ovarian isotransplantation from the fertile sister to their sibling who had POF, which offered a fascinating model beyond the mainstream of autotransplantation after ovarian cryopreservation (Silber et al. 2005, 2008, Silber & Gosden 2007). Extension of this technique and its application to ovarian homotransplantation (allografts) are probable developments in fertility preservation, because gamete and embryo donations are now mature and organized practices (Woodruff 1960; ASRM Practice Guidelines 2006, Guidelines for Gamete and Embryo Donation, http://www.asrm.org/detail.aspx?id=131).

Recent success has been reported with ovarian allografts in recipients who suffer from Turner’s syndrome, because they can restore pubertal development (Mhatre et al. 2005). However, ovarian allografts are still relatively rare, and the processes of immune surveillance are unclear when immune privilege is absent (Gosden 2007). With the lack of extensive human and animal studies, the rejection immunity involved in ovarian allografts remains largely unexplored. Traditional transplant studies of rejection and immunity have been predominantly undertaken in vitro or in vivo with extensive, tailored sample collection in either animal or human models. With the advent of in vivo bioluminescent imaging (BLI), luciferase reporter genes have been used widely in various biomedical fields for the non-invasive investigation of the cellular molecular events involved in normal and pathological processes (Zinn et al. 2008).
Luciferase-transgenic mice have been used as highly useful donors for monitoring hepatocyte, islet and stem cell transplantation (Yang et al. 2003, Fowler et al. 2005, Min et al. 2006). Realizing the full potential of this system may greatly benefit the study of the physiological behaviour and function of transplanted grafts.

Most cells infiltrating the allografts express T-cell markers, and are generally a mixture of CD4+ and CD8+ cells. The ratio of CD4+/CD8+ cells may vary in early versus late acute rejection (Hoshinaga et al. 1984, Ibrahim et al. 1993). CD19 is expressed earlier during B-cell development, and plays important roles in the peripheral immune system of diverse B-cell humoral transplant rejection (Yazawa et al. 2005).

We used optical molecular imaging, which expedites the fast-throughput screening of grafts, in a longitudinal study to monitor transplanted ovarian iso- and allografts in a comprehensive pilot model.

Results

The BLI photon quantity declined significantly below baseline in groups C and D from day 9 after transplantation, with no significant cyclic fluctuation. The most significant decline occurred in group C, all the way down to day 15. When the patterns of groups A and B were compared, there was a cyclic fluctuation every 4 days, but they were deeper in group A and only slight in group B, which presented the oestrus cycle as mild graft rejection and ageing. The trend in group E was relatively higher than that in group B, with cyclic fluctuation, but the difference was not statistically significant.

In vivo monitoring of ovarian graft bioluminescence revealed isograft survival for as long as 56 days in groups A, B and control group E. However, the photon quantity was much higher in groups B and E (Fig. 1A). Groups A,

![Graph](Image)

Figure 1 (A) Typical pattern of the representative engraftment of a mouse ovarian iso/allograft in each individual of each subgroup using in vivo BLI. The early loss of BLI indicated acute rejection at day 15 in group B and day 19 in group C. Longer survival of the graft, for up to 56 days after transplantation, in groups A, B and E indicated a lower rejection effect. (B) Typical trend in the photon quantity from the ovarian iso/allograft in each subgroup over time.
B and E showed fluctuating patterns of BLI engraftment with the oestrus cycle and follicle development as assayed by vaginal cytology and histology respectively (Fig. 1B). A complete loss of bioluminescence was noted on day 17 in group C (without cyclosporine A (CsA) treatment) and day 19 in group D (with CsA treatment). Similar to the BLI changes reflecting this engraftment pattern, histological analysis revealed extensive fat necrosis, myxoid degeneration and fibrosis, with no follicle presence, in groups C and D on day 19.

Peripheral T-cell subpopulations, examined across time, revealed lower CD4<sup>+</sup>/CD8<sup>+</sup> ratios and significant declines in groups C and D (allografts) compared with those in groups A and B (isografts; P<0.0001; Fig. 2A and B), indicating the development of acute rejection throughout the third week in the allografts. Peripheral CD19 increased, and was significantly higher between day 1 and day 20 in groups C and D than in groups A and B (P<0.001). NOD-SCID mice lacking T- and B-cells were used as the control.

Immunohistochemical analysis of CD4<sup>+</sup>/CD8<sup>+</sup> cells in the ovarian graft showed weak and no staining in groups A and B respectively from day 1. CD4<sup>+</sup>/CD8<sup>+</sup> cell infiltration of the ovarian graft produced mild staining on day 1 in group C but strong staining on days 8, 12 and 20 after transplantation in groups C and D (Fig. 3A–F, Table 1). Group E was always used as the control, with no CD4<sup>+</sup>/CD8<sup>+</sup> cell infiltration.

Consistent with the fluctuations in BLI, the ovarian follicles remained in groups A, B and E on day 56 but had vanished by day 19 because of fat necrosis in the grafts in groups C and D. Hormone activity revealed the presence of all four stages of the oestrus cycle, which is consistent with follicular development, in groups A, B and E. Compared with the fresh ovarian fragment and control group E, which showed various stages of follicular development, group E showed significantly lower numbers of follicles than the fresh control on day 0 but statistically more than groups A and B, and the primordial follicle count was higher in group B than in group A 56 days after transplantation (Fig. 4A–C and Table 2). Generalized myxoid degeneration and fat necrosis was noted on day 19 in groups C and D.
necrosis were observed, without any follicles, in groups C and D 19 days after transplantation. However, only the dioestrus stage was completed after transplantation in groups C and D.

**Discussion**

With the application of fertility preservation, reproductive endocrine glands may resume their function without hormone replacement therapy, and females may regain their fertility after adequate gonad preservation or donation. The orthotopic ovarian allotransplantation used for Turner’s syndrome restores the development of secondary sexual characteristics and menstruation, which echoes a series of ovarian isotransplantations in monozygotic twins (Mhatre et al. 2005, Silber et al. 2005, 2008, Silber & Gosden 2007). Regardless of the ethical issues, effective chronic immunosuppressant treatment warrants further study in this animal model.

Fertility is an important criterion by which women are judged, and ovarian transplant technology has the potential to reduce culturally entrenched discrimination against infertile women (R Nidamboor, February 2003, Science in Africa, www.scienceinafrica.co.za/2003/february/ovary.htm).

In contrast to ovarian isografts, which constitute a minority of ovarian grafts, ovarian allografts can be used in a wide range of patients with gonad failure, including gonadal dysgenesis, POF, surgical castration and chemotherapy/radiation-induced damage. The success of allotransplantation depends on rejection immunology, which varies with tissue differences, human leukocyte antigen (HLA) typing and immunosuppressants (Sayegh & Turka 1998, Cjte et al. 2001, Pidwell & Burns 2007). If all other factors are optimal, such as donor management, functional state of the donor ovary, surgical procedures and intraoperative recipient management, the main cause of transplant failure is rejection.

Ovarian graft size and longevity have never been quantified in vivo in the same individuals. This in vivo monitoring model is the first model used to investigate ovarian iso- and allografts systemically and longitudinally using a real-time optical imaging.

BLI has become an important component of biomedical research. It is ideal for small animal models because it can monitor biological processes with great sensitivity, efficiency, economy and versatility (Zinn et al. 2008). The advantages of in vivo imaging over in vitro imaging include its high throughput, lack of radiation, easy operation, functional analysis, constant monitoring, and real-time monitoring.

**Table 1** Profile of immunohistochemical staining for infiltrating CD4⁺/CD8⁺-positive cells in ovarian grafts over time.

<table>
<thead>
<tr>
<th>Group</th>
<th>T-cell</th>
<th>D1</th>
<th>D8</th>
<th>D12</th>
<th>D20</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVB</td>
<td>CD4/CD8</td>
<td>++/++</td>
<td>+++</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>FVB + CYC</td>
<td>CD4/CD8</td>
<td>--/--</td>
<td>--/-</td>
<td>--/-</td>
<td>--/-</td>
</tr>
<tr>
<td>Balb/c</td>
<td>CD4/CD8</td>
<td>++/++</td>
<td>++/++</td>
<td>++/++</td>
<td>++/++</td>
</tr>
<tr>
<td>Balb/c + CYC</td>
<td>CD4/CD8</td>
<td>++/++</td>
<td>++/++</td>
<td>++/++</td>
<td>++/++</td>
</tr>
<tr>
<td>NOD–SCID</td>
<td>CD4/CD8</td>
<td>--/-</td>
<td>--/-</td>
<td>--/-</td>
<td>--/-</td>
</tr>
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(++, strong stain; (+), weak stain; (−), no stain.)

![Figure 4](https://www.reproduction-online.org)

**Figure 4** (A) Fresh control ovarian primordial follicles (arrow) and primary follicles (arrowhead; magnification, ×400). (B) Ovarian primordial follicles in group A (arrowhead) and renal tubules (arrow; magnification, ×400) 56 days after transplantation. (C) Ovarian primordial follicles in group B (arrowhead) and renal tubules (arrow; magnification, ×400) 56 days after transplantation. (D) Typical pattern of generalized myxoid degeneration, with no follicles (magnification, ×400) in groups C and D 20 days after transplantation.
low variation, 3Rs (replacement, reduction and refinement), time efficiency through real-time imaging, digital quantum quantity in as little as 10 min by one researcher and the sacrifice of fewer mice.

In this study, we used transgenic donor mice carrying only a 712-bp luciferase transgene as the reporter, to create a significant genetically identical and non-identical model. The imaging technology has advanced to the point where it can rapidly reveal the fates of ovarian grafts, which have previously been elusive. The different recipient strains had the same haplotype as the FVB wild-type recipients or a different haplotype (Balb/C, allotransplantation) from that of the transgenic donor strain (FVB strain). Routine HLA typing for solid organ transplantation in humans has been concerned with the prognosis of chronic rejection. Therefore, we also used CsA, the most commonly used immunosuppressant, to test its suppression of ovarian rejection in this model (Sheldon & Poulton 2006).

Ovaries are not immunologically privileged. It is reasonable to use more aggressive and multiple chronic immunotherapies for allografts (Parkmann 1988, Paul 2001). The transplanted ovarian tissue represents a continuous source of HLA alloantigens capable of inducing a rejection response at any time after transplantation. Because this cannot be eliminated, the allograft continuously activates the T- and B-cell immune system, resulting in the lifelong overproduction of cytokines, constant cytotoxic activity and sustained alteration of the graft vasculature (Bellanti & Kadlec 1985, Bellanti & Rocklin 1985, McDevitt 1985, Roitt et al. 1985, Sompayrac 1999). The ovarian isografts, with or without CsA treatment, showed reasonably longer survival for up to 56 days with in vivo monitoring. CsA treatment alone was not effective against the rejection of ovarian allografts, which showed a limited longevity of 20 days.

Bioluminescence showed the complete rejection of allografts, with or without CsA, in 20 days. The absence of an oestrous cycle indicated the early loss of function. Ovarian isografts survived functionally for more than 56 days, but stronger luminescence also indicated a better outcome after CsA treatment. This study is consistent with previous reports that showed allografts, in contrast to isografts, were completely rejected in 20 days (Gosden 2007).

Allogeneic ovarian transplantation is not immunoprivileged, and will not succeed unless the recipient immune system is down-regulated. Furthermore, this downregulation by immunomodulation must be maintained on a lifelong basis because antigens (the allograft) are not self-limiting in solid organ transplantation, but are always present. The continued introduction of new immunosuppressants will provide greater access to transplantation (Bellanti & Kadlec 1985, Bellanti & Rocklin 1985, McDevitt 1985, Roitt et al. 1985, Sompayrac 1999).

Non-obese diabetic (NOD)–severe combined immunodeficient (SCID) mice, used as the control group for long-surviving ovarian grafts, are deficient in B-cell and T-cell functions, and lack detectable B-cells and pre-B-cells. However, they have normal numbers of natural killer cells, the main effectors of non-major histocompatibility complex-restricted immunity, which also includes the recognition of haemopoietic histocompatibility antigens on bone marrow allografts. Immune responses other than those involving T-cells and B-cells warrant further study.

In conclusion, transplantation models using luciferase-based imaging have revealed that in vivo monitoring with BLI of an ovarian transplant can detect changes in the functional mass resulting from immunological events, which ultimately affect the quality of the graft function and could potentially be used to test new immunosuppressants.

**Materials and Methods**

**Care and use of animals**

The 8-week-old FVB/N-TgRNA polymerase II (PolIII–Luc)Ltc transgenic donor mouse, H-2 haplotype (H^b_2), was created using the transgenic services of Level Biotechnology Inc., Hsi-Chih City, Taiwan (Fig. 5A). The construct (gene fragment) PolIII–Luc contains the 712-bp mouse PolIII promoter and a modified firefly luciferase cDNA (Promega pGL-2; Fig. 5B and C). This transgenic line was created using pronuclear microinjection of the PolIII–Luc transgene into fertilized FVB/N embryos at the Level Transgenic Center. Transgenic founder cells expressing the transgene were analysed for their expression patterns and tested for their germline transmission (Fig. 5C). The transgenic line was maintained as hemizygotes. An inbred strain of 8-week-old FVB/NJNarl wild-type mice with the H-2 haplotype (H^b_2) and 8-week-old Balb/cByNarl wild-type mice with the H-2 haplotype (H^b_2) was obtained from the National Laboratory Animal Center, Taiwan, ROC. Another control recipient 8-week-old Fox Chase female NOD mice–SCID mice (C.B-17 SCID) were obtained from BioLASCO, Taipei, Taiwan.
All the mice were housed under a 12 h light:12 h darkness regime at 22–24 °C, with food and water supplied without restriction. All procedures described here were reviewed and approved by the Animal Experimental Committee at the National Defense Medical Center and Tri-Service General Hospital, Taipei, Taiwan, ROC, in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

**Operative designs**

The mice were treated with ketamine (50 mg/kg) and xylazine (15 mg/kg) given i.p. to induce general anaesthesia. Donor mice (8 weeks old, sexually mature) and recipient mice (8 weeks old, sexually mature) underwent bilateral ovariectomy 1 week before transplantation. The recipient mice included group A (n = 5, FVB/N wild-type mice with the H-2 haplotype [H\textsubscript{2}]) (isogenic) without daily CsA treatment); group B (n = 5, FVB/N wild-type mice with the H-2 haplotype [H\textsubscript{2}]) with daily 25 mg/kg CsA treatment); group C (n = 5, Balb/c wild-type mice with the H-2 haplotype [H\textsubscript{2}]) (allogeneic) without daily CsA treatment); group D (n = 5, Balb/c wild-type mice with the H-2 haplotype [H\textsubscript{2}]) with 25 mg/kg CsA treatment); and group E (n = 5, NOD–SCID mice). The donor ovaries were divided into several fragments, followed by transplantation into the unilateral sub-renal capsule of each recipient strain (Fig. 5E). The mice in groups B and D were given CsA (25 mg/kg per day i.p.), starting 1 day before transplantation until they were killed. Another paired recipient group was processed under the same conditions for in vitro immunohistochemistry.

**In vivo BLI (in vivo tracking of viable cells and tissue)**

The mice were anaesthetized before injection with luciferin, and the transgene expression was measured (Fig. 5A). Reporter genes can be used to assay the activity of a particular PolII promoter in each viable cell. The reporter gene product is the...
luciferase enzyme. Luciferase catalyses a reaction with luciferin, producing light. The reporter gene product is simply placed under the control of the target promoter (PolII), and the activity of the reporter gene product is measured. Luciferin (Xenogen Corporation, Caliper Life Science, Alameda, CA, USA; catalogue #XR-1001) was prepared freshly in PBS (15 mg/ml) and sterilized by filtration. FVB/N-Tg(PolII–Luc)Ltc animals were injected i.p. with luciferin (150 mg/kg) 10 min before imaging, anaesthetized (1–3% isoflurane) and placed in a lightproof camera box. The mice were imaged dorsally for 3 min at high-resolution settings with a field of view of 20 cm. The light emitted from the transgene was detected with the IVIS Imaging System 50 Series (Caliper Life Science), digitized and displayed on a monitor. Living Image software (Caliper Life Science) takes data from the camera and displays the information on a pseudocolour scale, with colours representing the variations in signal intensity. The signals were quantified and archived using the Living Image software. Photons from the kidneys were quantified from the dorsal images using oval regions of interest (ROIs) of 3.5 x 3.5 cm² centred over the kidneys (Fig. 5D). The absolute intensity calibration of the IVIS Imaging System 50 Series was accomplished with a calibrated eight-inch integrating sphere (OL Series 425 Variable Low-Light-Level Calibration Standard, Optronic Laboratories, Inc., Orlando, FL, USA). We measured the ROI by imaging the counts detected by the CCD camera digitizer on the integrating sphere, which were converted to physical units of radiance in photons/s per cm² per steradian (Rice et al. 2006). Background signals were evaluated daily, and background subtraction calculations were performed with the Living Image software.

Subpopulations of CD4⁺/CD8⁺-positive T-cells and CD19⁺-positive B-cells in mouse blood detected by flow cytometry

Peripheral blood cells were collected and stained with phycoerythrin-conjugated anti-mouse CD8 antibody (Ab), allophycocyanin-conjugated anti-mouse CD4 Ab, and FITC-conjugated anti-mouse CD19 Ab (BD Bioscience Pharmanualing, San Jose, CA, USA; Riccardo et al. 2005). The cells were detected with flow cytometry on days 1, 5, 12 and 20 after ovarian graft transplantation.

Immunohistochemistry of CD4⁺/CD8⁺ cells in the ovarian graft

MABs labelled by the streptavidin–biotin (LSAB2; Dako Corporation, Santa Barbara, CA, USA) method were used for immunohistochemistry. The antibodies used were directed against CD4 (NeoMarkers, Fremont, CA, USA) and CD8 (CD4 Ab8, CD8 Ab1; NeoMarkers; Sato et al. 2005). Each Ab was tested on ten pairs of sections, and the staining was scored as negative, weak or strong, according to the staining intensity. The intensity scores were graded from weak to strong (−, none; +, weak and ++, strong staining).

Detection of oestrus cycle

Vaginal smears from the mature virgin female wild-type recipient mice (8 weeks old) were collected twice daily. The normal oestrus cycle of a laboratory mouse is 4–6 days in length, and has four stages: pro-oestrus, oestrus, metoestrus and dioestrus (Kimmin et al. 2004, Staley & Scharfman 2005). Cycle length was determined as the length of time between two consecutive occurrences of oestrus in animals that had progressed through at least three consecutive oestrous cycles. A 12 h light:12 h darkness cycle was maintained automatically, with lighting changes occurring at 0600 and 1800 h.

Ovarian follicles

Ovarian cortex biopsies involved cutting the samples into 5-μm sections, fixing them in 10% formalin, and staining them with haematoxylin and eosin in paraaffin sections to examine the various stages of follicular development. The primordial follicle count was measured in ten consecutive sections.

Statistical analysis

Light was quantified in terms of the proportional change from baseline (day 1). The peripheral T-cell subpopulation and B-cell activation were also assayed over time. To test the statistical significance of differences, ANOVA was used at each time point, with multiple comparisons adjusted using the Scheffe method. All analyses were performed with SAS 8.0, and P<0.05 was considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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