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ARTICLE

Evaluating the effects of immunosuppression by in-vivo bioluminescence imaging after allotransplantation of ovarian grafts

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Abstract Bioluminescence imaging (BLI) has been introduced for studies of ongoing biological processes but has never been applied for ovarian transplantation. Here, BLI was used as a novel approach to trace the survival of ovarian grafts. The ovarian donors were transgenic mice carrying *FVB/N-Tg (PolIII-luc)* as a reporter gene, encoding luciferase to catalyse luciferin which results in visible light emission as bioluminescence. There were three groups of recipients: (i) group A: BALB/c mice without immunosuppressant treatment; (ii) group B: BALB/c mice receiving a cocktail immunosuppressant treatment; and (iii) group C: immunodeficient NOD-SCID mice without immunosuppression. Luciferin BLI was used to follow graft survival, and viable follicle numbers were counted as a measure of success. Bioluminescence intensity fluctuated but was consistent with the end-point counts of viable follicle numbers. Group A showed loss of viable follicles and bioluminescence disappeared as early as day 10 following ovarian engraftment, indicating strong immune rejection. Groups B and C showed graft survival and measurable bioluminescence for up to 30 days. In conclusion, BLI provided non-invasive longitudinal dynamic monitoring of ovarian grafts with excellent sensitivity and spatial resolution. This approach should prove valuable for research on ovarian transplantation. 

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Introduction

Fecundity is essential for the propagation of any species and is always an issue of concern for gynaecologists. In the past few decades, scientists have made major efforts to solve human infertility problems. Premature ovarian failure – the loss of function of the ovaries before age 40 – has been estimated to affect 1% of the population (Coulam et al., 1986). In patients undergoing cancer treatment, radiotherapy and chemotherapy can improve their survival rate, but these treatments are also gonadotoxic. Some forms of ovarian failure are also attributed to autoimmune disorders, metabolic disorders (galactosaemia), infectious diseases (e.g., mumps) and genetic disorders such as Turner syndrome and Fragile X syndrome (Goswami and Conway, 2005; Laml et al., 2000). For patients with premature ovarian failure or undergoing cancer therapy, the focus has been on the cryopreservation of oocytes or ovaries for subsequent auto-transplantation (Bromer and Patrizio, 2009; Donnez et al., 2009; Kim, 2006; Kim et al., 2001; Kuwayama et al., 2005; Poirot et al., 2007; Song et al., 2009). Unfortunately, for patients with autoimmune or genetic disorders, gonadal auto-transplantation is ineffective in preserving fertility. In such cases, allotransplantation might be a remedy. Silber and colleagues reported a series of monozygotic twins who underwent ovarian iso-transplantation to rescue the sterile sister (Silber, 2009; Silber et al., 2005, 2008). Furthermore, orthotopic ovarian allotransplantation has been performed on patients diagnosed with Turner syndrome. This restored regular menstruation and ovulation, raised hormonal concentrations and led to the development of secondary sexual characters (Mhatre and Mhatre, 2006; Mhatre et al., 2005).

The present state of transplant biology, as both a basic and a clinical science, is unlikely to have been achieved without lessons learned from rodent transplant models. Progress in this area has occurred in several ways over the last few decades, including organ preservation, overcoming ischaemia/reperfusion injury and immune rejection and the development and testing of new immunosuppressive regimens. Many efforts have been made to evaluate possible rejection and survival models of solid organ transplantation and to generate monitoring systems for tissues such as the pancreatic islets, kidney, liver and heart (Berman et al., 2009; Martinez-Dolz et al., 2009; Shapiro et al., 2000; Truong et al., 2009). However, there are few studies addressing the pathophysiology of ovarian allografts, which are indispensable in the development of ovarian transplantation (Chen et al., 2010). With respect to study design, in-vivo models have more advantages than in-vitro ones. An in-vivo non-invasive approach saves not only the cost of experiments but also the lives of experimental animals. In addition, it eliminates intra-individual differences and allows 'natural' systemic follow-up after transplantation. For this purpose, bioluminescence imaging (BLI) provides a technique that is capable of excellent sensitivity and spatial resolution but non-invasive monitoring of ovarian grafts.

The main objectives of this study were to apply BLI to tracking ovarian survival after allotransplantation and to compare this measure with the analysis of viable follicular survival.

Materials and methods

Care and use of animals

A transgenic mouse line, Tg (*PolIII-luc*), on an FVB/N (H_2^q) background, was generated by the transgenic service of Level Biotechnology (Taipei County, Taiwan) and used as ovarian donors. The FVB/N-Tg (*PolIII-luc*) Ltc transgenic mouse strain with a H_2^q haploid genotype carries *PolIII-luc* as a targeting gene fragment encoding a 712-bp mouse RNA polymerase II promoter (*PolIII*) and modified firefly luciferase cDNA (pGL-2; Promega, Madison, WI, USA). Generation of the germ line was mediated by microinjection of the *PolIII-Luc* transgene into the pronuclei of FVB/N zygotes. The animals were maintained as hemizygotes that express the transgene for luciferase (*luc*) and have been tested for germline transmission. The inbred BALB/cByJNarl wild-type mouse strain with a H_2^d haploid genotype was obtained from the National Laboratory Animal Centre (Taipei City, Taiwan). Another recipient, Fox Chase female non-obese diabetic (NOD) severe combined immunodeficient (SCID) mouse strain (C.B-17 SCID), was obtained from BioLASCO Taiwan (Taipei City, Taiwan).

All mice in this study were bred in the animal house of the National Defense Medical Centre and housed under a 12/12 h light/dark regimen at 22–24 °C, with food and water *ad libitum*. All procedures adhered to the Guiding Principles for the Care and Use of Laboratory Animals and were reviewed and approved by the Animal Experimental Committee at the National Defense Medical Centre and Tri-Service General Hospital (Taipei City, Taiwan).

Study design

All mice were 5 weeks old and sexually mature. The recipients' gonads were removed 1 week before transplantation. The ovarian donors were FVB/N-Tg (*PolIII-luc*) Ltc transgenic mice. The recipients were allogeneic wild-type BALB/c and NOD-SCID mice. The recipients were classified into three groups ($n = 6$ each). Group A comprised BALB/cByJNarl mice without immunosuppressive treatment. Group B was the same strain treated with a multi-immunosuppressive regimen. This included oral CellCept (mycophenolate mofetil, daily single dose of 20 mg/kg; Roche, Taiwan), oral Imuran (azathioprine, daily single dose of 20 mg/kg; GlaxoSmithKline, USA), intra-peritoneal injections of cyclosporin (daily single dose of 20 mg/kg; Novartis, Switzerland) and Solu-Medrol (methylprednisolone, daily single dose of 1 mg/kg; Pfizer, USA). The regimens were given as one dose and 1 day before transplantation and then a single dose every day for 30 days. Group C was immunodeficient NOD-SCID mice without immunosuppression. Each transplanted ovarian graft measured $1.5 \times 1.5 \times 1.5$ mm. After washing in phosphate-buffered saline (PBS), the grafts were implanted under the subrenal capsule of each recipient. All transplanted grafts were monitored by in-vivo BLI for 30 days and compared with the quantitative histological assessment of follicular number on day 30.

Immunosuppressive regimens

CellCept has been used in humans for prophylaxis against rejection after cardiac, liver and renal transplantation (Knight et al., 2009; Woodroffe et al., 2005). It produces potent, non-competitive inhibition of inosine monophosphate dehydrogenase and thus blocks de-novo synthesis of guanosine nucleotides. Because lymphocytes depend upon the de-novo pathway for purine synthesis, the proliferative responses of T- and B-lymphocytes, as well as antibody formation and the generation of cytotoxic T-cells, are inhibited. In contrast, the purine analogue Imuran produces competitive inhibition of purine synthesis and interferes with the synthesis and function of RNA and DNA. It has been used for preventing renal transplant graft rejection (Knight et al., 2009; Woodroffe et al., 2005). Cyclosporin has shown synergism with CellCept. Its actions are dependent upon binding to intracellular sites of action. The intracellular protein most closely linked to the immunosuppressive activity of cyclosporin is cyclophilin. By binding to cyclophilin, the antigenic response of helper T-lymphocytes is inhibited and the production of interleukin-2 and interferon- γ is suppressed. Solu-Medrol is a synthetic glucocorticoid with multiple mechanisms of action to modify the body's immune responses, including anti-inflammatory activity, immunosuppressive properties and anti-proliferative actions. The immunosuppressive properties decrease the response to delayed and immediate hypersensitivity reactions (e.g., types III and IV). The access of sensitized T-lymphocytes and macrophages to target cells can also be prevented by corticosteroids.

In-vivo bioluminescence imaging

The ovarian donors, FVB/N-Tg (*PolIII-luc*) Ltc transgenic mice, expressed *PolIII-luc* as the reporter gene under the control of the RNA polymerase II promoter (*PolIII*). The reporter gene encodes the enzyme luciferase that catalyses chemical reactions of molecular oxygen with the substrate (luciferin). This reaction generates visible light from viable cells that can be measured quantitatively. Luciferin (no. XR-1001; Xenogen, Alameda, CA, USA) was diluted in PBS (15 mg/ml) and sterilized by filtration. After anaesthesia with 1–3% isoflurane, the recipient mice were injected with luciferin intraperitoneally (150 mg/kg) 10 min before imaging. Fur will auto-fluoresce and may mask the bioluminescence signal. Therefore, 2 h before the imaging was obtained, the skin was shaved. The mice were then placed in the prone position in a light-tight imaging chamber and imaged for 3 min from the dorsal side at high-resolution settings. Bioluminescence imaging was performed using a cooled charge-coupled device (CCD) camera on an IVIS imaging system 50 series (Xenogen). Using IVIS Living Image software (Caliper Life Sciences, Hopkinton, MA, USA), the image from the camera was digitized using a pseudocolour scale with colours representing different signal intensities. A round region of interest (ROI; 3.0×3.0 cm) was selected on the dorsal trunk and luminescence was quantified by summing pixel intensities within the ROI using a calibrated 8-inch integrating sphere (OL Series 425 Variable Low-Light-Level Calibration Standard; Optronic Laborato-

ries, Orlando, FL, USA). The quantity unit (p/s) of the IVIS software was adjusted by the calibrated 8-inch integrating sphere to avoid bias during the different parameter set-up. The integrating sphere is illuminated with a tungsten lamp, and the light level entering the sphere can be stable. IVIS software uses it as a reference to calibrate the light intensity from any parameter set-up changing of the CCD camera. That is why absolute intensity calibration of the CCD camera and the overall imaging system is necessary to calculate brightness (in physical units) of luminescent cells and to estimate the number of cells inside an animal from the intensity of the surface image (Rice et al., 2001). Serial background signals were evaluated daily for 30 days and the IVIS software was used to subtract background light.

Tissue collection and histology of ovarian follicles

On day 30 after transplantation, the ovarian engraftments were removed and fixed in 10% formalin and 5- μ m-thick paraffin wax sections were stained with haematoxylin and eosin. Randomized five sections were studied from each recipient and the mean numbers of viable follicles were counted for each group. The follicles were classified into primordial follicles surrounded by one layer of squamous granulosa cells, and growing follicles, including primary to tertiary follicles, surrounded by a zona pellucida, one to multiple layers of cuboidal shape granulosa cells.

Statistical analysis

Photons were quantified and measured as proportional changes over time from baseline (measurement at a certain day – measurement at baseline)/measurement at baseline). To test whether the differences among the three groups were statistically significant, analysis of variance (ANOVA) was used at days 1–10 with multiple comparisons adjusted by Scheffe's method. Differences between groups B and C at day 10 after transplantation were compared using Student's *t*-test. All statistics were processed using SAS version 9.0 (SAS Institute, Cary, NC, USA) and $P < 0.05$ was considered statistically significant.

Results

There was gradual attenuation of bioluminescence in group A and complete loss by day 10 after transplantation (Figures 1A and 2). In group B, with multi-immunosuppressive medication, bioluminescence decreased initially on day 2, then increased between days 2 and 4 with a subsequent cyclic fluctuation in intensity and declined again from day 8 to a very low level on day 12. However, there was measurable bioluminescence output to day 30. In group C, with immunodeficient NOD-SCID mice, the bioluminescence showed an initial increase, then showed an upward trend between days 4 to 10, followed by attenuation to a very low level after day 12. It persisted to day 30 after transplantation with milder fluctuations. The most important change observed in group A without anti-rejection treatment was the loss of bioluminescence after day 10. Statistically, group A was significantly different from group B and group C (Table 1) where

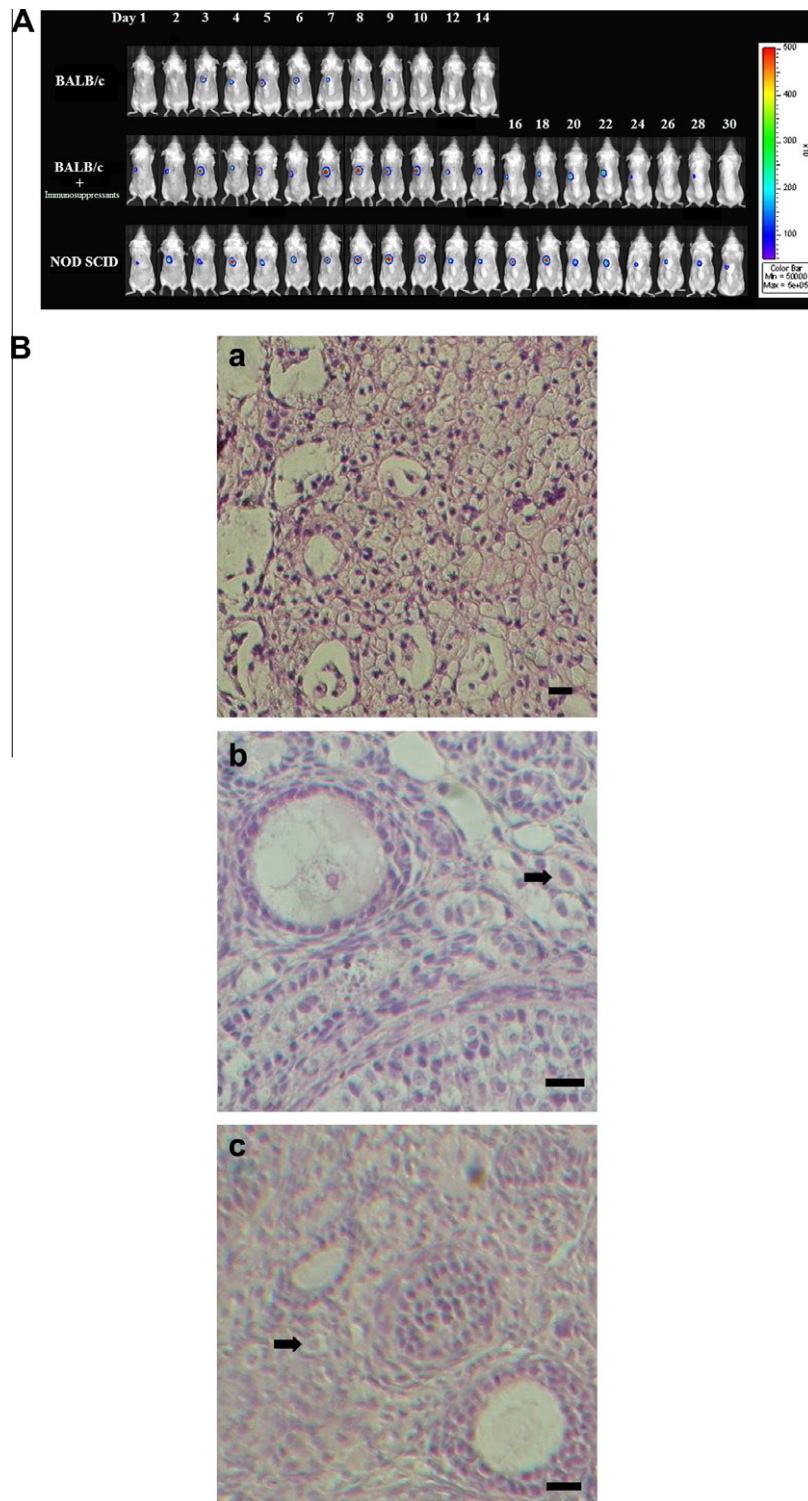


Figure 1 (A) Longitudinal tracking of the ovarian graft after transplantation by in-vivo bioluminescence imaging. BALB/cByJNarl mice without immunosuppressive treatment (group A) revealed gradual attenuation with complete loss by day 10 after transplantation. BALB/cByJNarl mice treated with a multi-immunosuppressive regimen (group B) and immunodeficient NOD-SCID mice without immunosuppression (group C) maintained a detectable ovary for 30 days. (B) Histological assessment on day 30 after transplantation. a: group A demonstrating extensive fat necrosis without viable follicles. b, c: groups B and C showing viable follicles. Arrow = primordial follicle. Bar = 20 μ m.

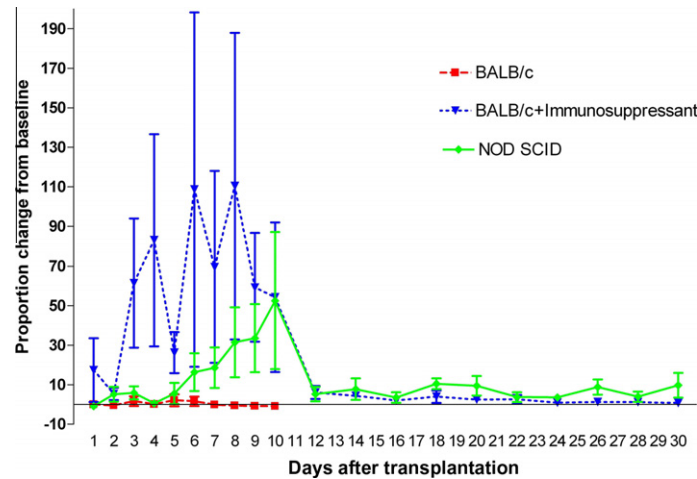


Figure 2 The trend of digitized photon intensity. BALB/c mice without immunosuppressants (group A) showed complete loss of detection after day 10. BALB/c mice given immunosuppressants (group B) showed cyclic fluctuations and NOD-SCID mice (group C) demonstrated a decline after day 10. Both groups B and C declined to a low level on day 12 but maintained detectable bioluminescence to day 30 after transplantation.

Table 1 Comparison of bioluminescence intensity by ANOVA from days 1–10 after transplantation.

Day	Group comparisons		
	A versus B	A versus C	B versus C
1	−17.4 (−27.3 to −7.5) ^a	0.9 (−9.0 to 10.8)	18.3 (10.8 to 25.8) ^a
2	−5.9 (−8.9 to −2.9) ^a	−5.7 (−8.7 to −2.7) ^a	0.2 (−2.1 to 2.4)
3	−59.7 (−79.9 to −39.6) ^a	−4.2 (−24.3 to 16.0)	55.5 (40.3 to 70.8) ^a
4	−89.9 (−115.9 to −50.0) ^a	−0.5 (−33.5 to 32.5)	82.4 (57.5 to 107.4) ^a
5	−24.1 (−31.3 to −16.8) ^a	−3.4 (−10.6 to 3.8)	20.7 (15.2 to 26.2) ^a
6	−107.1 (−162.6 to −51.6) ^a	−14.8 (−70.3 to 40.7)	92.3 (50.3 to 134.3) ^a
7	−67.7 (−100.2 to −39.2) ^a	−18.8 (−49.3 to 11.7)	50.9 (27.9 to 74.0) ^a
8	−111.0 (−160.0 to −62.0) ^a	−32.0 (−81.0 to 16.92)	78.96 (42.0 to 116.0) ^a
9	−60.0 (−80.0 to −40.0) ^a	−34.4 (−54.3 to −14.4) ^a	25.6 (10.5 to 40.7) ^a
10	−55.1 (−86.7 to −23.6) ^a	−53.4 (−84.9 to −21.9) ^a	1.8 (−22.1 to 25.6)

Values are mean difference (95% CI).

A = BALB/c without immunosuppressants; B = BALB/c + immunosuppressants; C = Fox Chase non-obese diabetic severe combined immunodeficient.

^a $P < 0.0001$. In group B, with immunosuppressive medication, the bioluminescence was significantly stronger than in groups A or C.

bioluminescence was lost after day 10. In group B, with immunosuppressive medication, the bioluminescence was significantly stronger than in groups A ($P < 0.001$ days 1–10) or C ($P < 0.001$ days 1, 3–9) (Table 1). In groups B and C, bioluminescence was low by day 12, but both groups maintained detectable signal intensity to day 30. However, group C showed significantly stronger signal intensities with mild fluctuations than did group B after day 12 ($P = 0.0049$ day 14, $P = 0.019$ day 16, $P < 0.001$ days 18, 20, 24, 26, 28 and 30; Table 2).

When follicle numbers were counted on day 30 after transplantation, the ovarian graft in group A showed no viable follicles, with complete fatty necrosis and fibrosis (Figure 1B, Table 3). On the other hand, the sections from

groups B and C demonstrated viable follicles corresponding to the detectable bioluminescence emission until day 30 (Figure 1B). As with the bioluminescence measure, the follicle numbers were significantly different between groups B and C at day 30 ($P = 0.023$ for number of primordial follicles, $P = 0.001$ for number of growing follicles and $P = 0.013$ for total follicle number; Table 3).

Discussion

Investigation of solid organ transplantation with the help of rodent models was developed for many decades before clinical trials commenced in humans. Surgical techniques and

Table 2 Comparisons of bioluminescence intensity between groups B and C after day 12.

Day	Group B versus group C	P-value
12	0.8 (−1.2 to 2.8)	0.4309
14	−3.4 (−5.8 to 1.5)	0.0049 ^a
16	−1.5 (−2.8 to −0.3)	0.019 ^a
18	−6.6 (−8.3 to −4.9)	<0.001 ^a
20	−7.1 (−9.3 to −4.9)	<0.001 ^a
22	−1.1 (−2.5 to 0.3)	0.1138
24	−2.7 (−3.7 to −1.7)	<0.001 ^a
26	−7.5 (−9.3 to −5.8)	<0.001 ^a
28	−2.8 (−4.1 to −1.6)	<0.001 ^a
30	−8.9 (−11.7 to −6.2)	<0.001 ^a

Mean difference (95% CI).

A = BALB/c without immunosuppressants; B = BALB/c + immunosuppressants; C = Fox Chase non-obese diabetic severe combined immunodeficient.

^aP < 0.05 (Students' *t*-test). The bioluminescence intensity was significantly different between groups B and C after day 12.

the survival of engrafted tissues have been discussed (Boros et al., 2007; Eimani et al., 2009). The cornerstone of graft survival is to suppress the immune response against the allograft that is being recognized as 'non-self,' which results in different types of rejection. Acute rejection usually develops after the first week, which is associated with incompatible human leukocyte antigen type and is mediated by T-lymphocytes. In richly vascularized organs, such as the kidney and liver, there is a greater risk for the development of acute rejection. For example, the incidence of acute rejection of the first kidney transplantation is 60–75%, and in orthotopic liver transplantation, it is 20–60%, with most episodes occurring on post-operative days 7–21 (Hubscher, 1996).

Following grafting revascularization commences within 24 h in the mouse (Schneider-Kolsky, 1997). Revascularization facilitates luciferin delivery to the ovary while the current study also showed accentuation from day 2 after transplantation. The data presented here demonstrate that bioluminescence in group A vanished after day 10. In group B, with immunosuppressant treatment, the decline began at day 8 with marked attenuation after day 10. In group C,

the bioluminescence also declined after day 10. Hence, it is inferred that acute rejection in this murine model of ovarian allotransplantation occurred around day 10. Compared with group A, the immunosuppressants administered to group B prolonged the graft survival but were insufficient to prevent acute rejection.

NOD-SCID mice have innate immune deficiency and lack T- and B-lymphocytes but have been reported to have normal natural killer cell activity (Dorshkind et al., 1985; Murphy et al., 1987). In this study, the bioluminescence intensity of group B was significantly stronger than that of group C before day 10 after transplantation. This difference suggests that some mechanisms associated with rejection in NOD-SCID mice were prevented by immunosuppressant treatment in group B. However, after day 10, acute rejection destroyed the ovarian graft in group B with significantly poorer survival than in group C. This is reasonable because T-cells are involved in acute rejection.

To monitor graft survival, a practicable instrument is needed to assay viable follicles as well as their actual function. Traditional studies with rodent models have utilized numerous samples by time course to meet statistical requirements. An approach by in-vivo imaging in contrast to in-vitro studies has the advantages of high throughput. Moreover, it is easy to operate, gives low variation and allows for continuous monitoring of functional status. There is no radiation; recording real-time digitally quantified images saves time and utilizes many fewer mice for time course studies. Other than current imaging techniques such as computed tomography, magnetic resonance or positron emission tomography, BLI demonstrates superiority with its excellent sensitivity and spatial resolution.

Bioluminescence imaging is one kind of genetically encoded imaging reporter system introduced into cells and transgenic animals. Through luciferase-mediated oxidation of luciferin, BLI enables non-invasive, longitudinal studies of dynamic biological processes in intact cells and living animals. BLI has been introduced into many aspects of basic research including monitoring post-transplantation grafts of hepatocytes, pancreatic islets and stem cells. These have shown the reliability and practicability of this approach (Dothager et al., 2009; Fowler et al., 2005; Virostko et al., 2009).

Other examples of these imaging reporters include fluorescent proteins with intrinsic signal production or direct binding or import of a radiolabelled reporter substrate or probe. The intensity and time course of reporter gene activity should correlate with the strength and duration of endogenous target gene expression and thus could provide a stable source of signal enabling longitudinal studies in living organisms with high temporal and, in some cases, high spatial resolution. With especially high signal-to-noise levels, BLI with luciferase reporters provides a relatively simple, robust, cost-effective and extremely sensitive means to image fundamental biological processes *in vivo*, as shown in this study. The fluctuation of photon intensity in these three groups provided reasonably accurate monitoring of the engraft survival, and the day-30 quantified photon intensity corresponded with the end-point analysis of viable follicle numbers in the grafted ovaries.

Because many processes of physiology and disease are dynamic in time and space, end-point assays do not always

Table 3 Follicle numbers of each transplant graft on day 30 after transplantation.

Follicle	Group			P-value
	A (n = 6)	B (n = 6)	C (n = 6)	B versus C
Primordial	0	529.7 ± 33.7	621.7 ± 29.0	0.023
Growing	0	80.3 ± 0.6	95.0 ± 3.0	0.001
Total	0	610.0 ± 33.8	716.7 ± 27.0	0.013

Values are mean ± standard deviation.

A = BALB/c without immunosuppressants; B = BALB/c + immunosuppressants; C = Fox Chase non-obese diabetic severe combined immunodeficient.

provide a comprehensive assessment. In this article, tissue biopsy with counting of follicular number on day 30 after transplantation could not reflect the fluctuating bioluminescence intensity, which represented dynamic destruction of the graft by post-transplantation immune rejection. In addition, although the eventual follicular number assessed by tissue biopsy revealed more follicles in the NOD-SCID mice on day 30, the use of a multi-immunosuppressant regimen improved allograft survival before day 10 as demonstrated by in-vivo BLI.

With BLI, biopsy every day to count the follicular number is not needed, but this study has sequential, longitudinal and dynamic tracking of graft viability. However, there are some limitations with BLI in monitoring ovarian transplantation. First, although it can help in determining graft survival, rejection and longevity, it cannot actually represent graft function without doing hormone assays from blood sampling. Vaginal cytology is an alternative for monitoring the oestrous cycle. Second, it is not known if this genetically encoded imaging reporter might influence the hormone profile of the allograft. Third, one study related to scar and BLI used novel transgenic mouse model with a Smad2/3-responsive luciferase reporter construct (SBE-luc) to report transforming growth factor β 1 expression that correlates with scarring which may influence in-vivo real-time imaging (Satterwhite et al., 2007). The surgical scar between the graft and the camera may have influenced the results, although each group shared the same effect. Therefore, more research is needed to ensure the practicability of BLI for any subsequent application in humans.

In conclusion, BLI was combined with end-point analysis to monitor ovarian grafts. Obviously, end-point assays are not sufficient to gain a complete understanding of disease pathogenesis because many of the steps toward development of a disease are dynamic in time and space. Advances in in-vivo BLI provide non-invasive, spatial, longitudinal tracking after ovarian transplantation, and thus it facilitates a more comprehensive understanding of existing problems. It can promote further study of therapeutic interventions that could improve clinical outcomes for women requiring ovarian allografting to restore their fertility.

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