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Tracing location by applying Emerald luciferase in an early phase of murine endometriotic lesion formation

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Abstract: The pathogenesis of endometriosis has not been fully elucidated. We focused on the behavior of the ectopic endometrium, that is, the origin of the endometriotic lesion, before adhering to the peritoneal cavity. To observe lesion formation in the very early phase, we developed a novel endometriosis animal model using bioluminescence technology. We established a new transgenic mouse that expressed Emerald luciferase (ELuc) under the control of the CAG promoter. This transgenic mouse, called the CAG-ELuc mouse, showed strong bioluminescence emission; we succeeded in tracing the lesion location by the emission of ELuc. The accuracy of tracing by ELuc was high (57.7–100% of correspondence) and depended on the dosage of E2 administration. In the very early phase after transplantation, the process of lesion formation can be observed non-invasively and chronologically. We have verified that the preferred location of the uterus (transplanted grafts) was fixed immediately after the transplantation of the grafts.
Key words: bioluminescence, endometriosis, *in vivo* imaging, mouse model, transgenic mouse

Introduction

Endometriosis is a serious disease in which the endometrium grows in extrauterine locations, mainly in the peritoneal cavity. The effects of endometriosis include inflammation, pain, tumors, and infertility. The prevalence of this condition ranges from 17% to 47% in infertile women and 2% to 74% in women with chronic pelvic pain [1]. In general, women with endometriosis have medical treatments, such as oral contraceptives (OC), progestins, and GnRH agonists, thereby relieving their endometriosis-associated pain and endometriotic lesion growth. These are symptomatic treatments. Although the pathogenesis of endometriosis remains unclear, many hypotheses have been proposed, such as retrograde menstruation, Müllerian remnants, apoptosis defects, epigenetics, and genetics. Sampson's "transplantation theory [2]" based on retrograde menstruation is plausible. A causal treatment, such as an approach to provide an environment in which the ectopic endometrium is hard to adhere to the abdominal cavity, is desirable.

Animal models of endometriosis have been developed to understand the disease mechanism. One such model uses baboons [3, 4] that have a menstrual cycle. It is expected that baboons are optimal to reproduce the pathophysiology of human endometriosis because they are phylogenetically similar to humans. However, these

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primates are costly and require a high degree of maintenance. Therefore, animal models using smaller mammals such as rodents are employed [5, 6], established by allotransplantation of the endometrium or xenotransplantation using immunodeficient rodent animals. Even though mice do not have a menstrual cycle as humans, it is possible to develop conditions resembling endometriosis [7].

The early period of the endometriotic lesion formation is not fully understood, but previous studies have demonstrated that various molecules are associated with the pathogenesis of endometriosis. Studies using murine models have reported that endometriotic lesion formation was suppressed by a gene knockout (KO). For example, the growth of endometriotic lesions was reduced in TGF-β1-KO mice [8], and the growth and number of endometriotic lesions were suppressed in PAR2-KO mice [9]. These animal models require histological analysis to confirm endometriosis-like lesions in the peritoneal cavity after sacrifice; therefore, it is difficult to observe the chronological progression of endometriosis in the same animal. Hence, it is unclear how the scattered ectopic endometrium develops as lesions in the peritoneal cavity and the fate of ectopic endometrium that does not develop as the lesion.

In vivo imaging system (IVIS) is a tool that can accomplish our purpose to unravel the chronology of the ectopic endometrium in the early period of lesion formation. IVIS can detect luminescence and fluorescence in living animal bodies non-invasively over time [10, 11]. We propose applying IVIS for an investigation into the early phase of endometriosis in animal experiments. The Green Mouse expresses green fluorescent protein (GFP) ubiquitously [12] and is known as a solid reporter mouse. However, it is considered that GFP are not suitable for non-invasive observation because it is difficult for GFP excitation light to reach the deep areas of the body and the wavelength of emission light is absorbed by tissues [13].

Bioluminescence technology does not require excitation light. Instead, administration of D-luciferin that is a substrate for the reaction of luciferase can be used. In our experimental model, the pieces of the uterus (grafts) that are transferred intraperitoneally to form endometriosis lesions are small (e.g., 1 × 1 mm). Therefore, a potent bioluminescence gene that can be detected non-invasively must be selected. Emerald luciferase (ELuc) is stable and has higher light emission in viable cells than firefly luciferase [14]. Additionally, the CAG promoter enables high and ubiquitous expression [15]. Therefore, when the CAG promoter is employed to drive Emerald luciferase, a high expression of Emerald luciferase would be expected.

In this study, we established a new reporter mouse using the Emerald luciferase gene driven by the CAG promoter; we evaluated whether the mouse could be suitable as an endometriosis animal model to examine graft migration taking place in the early phase of the endometriotic lesion formation.

Materials and Methods

The experimental protocol was approved by the Institutional Animal Care and Use Committee and the Safety Committee for Recombinant DNA Experiment at Tottori University.

Vector construction

Figure 1 shows the pCAGGS-ELuc vector used to generate the CAG-ELuc transgenic mouse. The Emerald luciferase (ELuc) gene is a reporter gene expressed by the CAG promoter. The pCAGGS vector contained the CAG promoter consisting of the CMV immediate-early (IE) enhancer, chicken beta-actin promoter, and rabbit beta-globin poly (A) signal [15], and was provided by the RIKEN BioResource Center through the National BioResource Project of the MEXT/AMED, Japan. The linker containing the *Not*I and *Eco*RV sites was inserted into the blunted *Eco*RI site of the pCAGGS vector. The ELuc gene was excised from pELuc-TEST (ELV-101, TOYOBO Co., Ltd., Osaka, Japan) by *Not*I and *Eco*RV, and inserted into the modified pCAGGS vector.

Generation of the CAG-ELuc transgenic mouse

The transgene was excised from the pCAGGS-ELuc vector using *Sal*I and *Avr*II (Fig. 1). The purified trans-

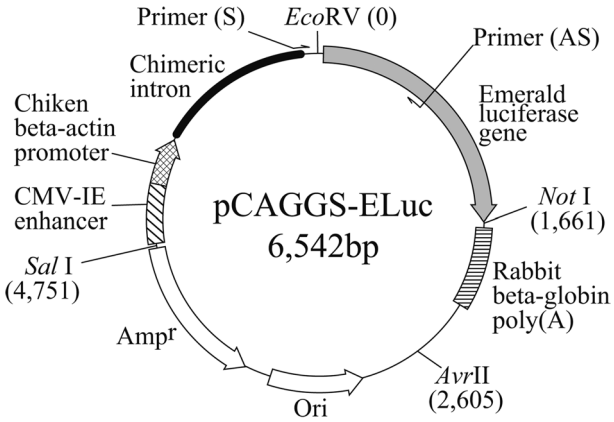

Fig. 1. The pCAGGS-ELuc vector construction for the CAG-ELuc transgenic mouse. The transgene was cut from *Sal*I to *Avr*II for microinjection. Primer (S) and Primer (AS) were used as genotyping primers to detect transgene. The pCAGGS-ELuc vector backbone was the pCAGGS vector.

gene aqueous solution (2.75 ng/μl) (W1503 water for embryo transfer, sterile-filtered, BioXtra, suitable for mouse embryo, SIGMA-ALDRICH Co., St. Louis, MO, USA) was injected into the pronuclei of fertilized zygotes obtained from the C57BL/6 strain. These zygotes were cultured until they developed into two-cell embryos. These embryos were then transferred into the oviducts of pseudo-pregnant ICR mice. The founder mice were confirmed by genomic PCR with KAPA2G Robust HS RM with dye (KK5706, NIPPON Genetics Co., Ltd., Tokyo, Japan) according to the instructions of manufacturer. The sequences of sense (S) and antisense (AS) primers for genotyping PCR were 5'-tacagctcctgggcaacgtgctgg and 5'-ccatgaaatagcccaggttgatactgaagc, respectively. ELuc expression from the transgene was confirmed by a luciferase assay. The cut tails of transgenic mice were soaked in 50 μl Emerald Luc Luciferase Assay Reagent Neo (ELA-301, TOYOBO Co., Ltd.) and added to a 96-well black plate (237105, Thermo Scientific™ Nunc™ F96 MicroWell™ Black Polystyrene Plate, ThermoFisher Scientific Inc., Waltham, MA, USA). The bioluminescence of ELuc was detected using PHELIOS (AB-2350, ATTO Corp., Tokyo, Japan). The exposure time was set to 1 s. To maintain this strain, C57BL/6J Jms Slc mice were used for mating.

In vivo and *ex vivo* imaging of the CAG-ELuc transgenic mouse

The CAG-ELuc female mice (17 week-old) were intraperitoneally injected with 150 mg/kg D-luciferin (126-05116, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). Ten minutes later, they were anesthetized with M/M/B anesthesia consisting of medetomidine (Domitor® Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan), midazolam (SANDOZ, SANDOZ K.K., Tokyo, Japan), and butorphanol (Vetorphale®, Meiji Seika Pharma Co., Ltd., Tokyo, Japan) at doses of 0.3, 4, and 5 mg/kg body weight, respectively. At fifteen min after D-luciferin administration, the bioluminescence of the whole body was detected using an IVIS® Lumina imaging system (PerkinElmer Inc., Waltham, MA, USA). The major organs were promptly excised after sacrifice by cervical dislocation and luminescence was measured. The exposure time was 0.2 s. Trunk hair was removed under anesthesia the day before the imaging.

Generation of endometriosis animal model

The CAG-ELuc female mice as donors and C57BL/6 female mice (wild type) as recipient were anesthetized with M/M/B anesthesia as described above. They were ovariectomized and injected with E2 (Estradiol valerate, Progynon®-Depot, Fuji Pharma Co., Ltd., Toyama, Japan, 0.5 or 0.2 μg/mouse, according to each experimental condition) subcutaneously. After surgery, they were awoken by administration of atipamezole (ANTISEDAN®, Nippon Zenyaku Kogyo Co., Ltd., 0.3 mg/kg body weight) that is antagonistic to medetomidine. One week later, the donor mice were sacrificed by cervical dislocation, and their uteri were removed. Half of the uterus was minced in 300 μl of saline. The minced uterine tissues were transferred into the peritoneal cavity of the recipient mice under anesthesia (i.e., 1:2 donor uterus to recipient ratio), and the surgery hole was sutured. Subsequently, the recipient mice were administered E2 (0.5 or 0.2 μg/mouse). After uterus transfer, E2 was injected into the recipient mice once a week.

Detection of the lesion location by *in vivo* imaging and autopsy

The mice of 41 to 49 day-old were used. In this experiment, two different doses of E2, i.e., 0.5 and 0.2 μg/mouse, were examined. *In vivo* imaging and anesthesia were performed as described above. Experimental Day 0 was set as the day of uterus transfer. The imaging exposure time was 10 s. The regions of interest (ROIs) were marked with a red square (Fig. 4a). The flux of luminescence in the square was recorded, and the mean of three ROIs of the abdominal, right, and left sides (Fig. 4a) was computed as the intensity of luminescence. Sequentially, their abdomens were opened after sacrifice, and the location of endometriosis-like lesions and pieces of the uterus was confirmed by autopsy. The correspondence rates between the location of bioluminescence and the actual location of grafts or lesions confirmed by macroscopic observation were calculated. Images of the removed lesions were taken using a stereomicroscope and *ex vivo* imaging by IVIS; the weight of lesions was recorded. These tests were performed on experimental Days 3 and 14.

Chronological and non-invasive *in vivo* imaging analysis of the endometriosis mouse model

The mice of 41 to 43 day-old were used. In this experiment, the E2 was 0.5 μg/mouse. This *in vivo* imaging was performed as described above on Days 1, 3, and 7. The exposure time was 10 s. The abdominal region was imaged *in vivo* using IVIS. The mice were sacrificed by cervical dislocation after the experiment.

Statistical analysis

All error bars in the graphs shows the standard deviation. All significant differences were tested using the Wilcoxon rank-sum test. The tests were performed using the "exactRankTests" package [16, 17].

Results

Generation of the CAG-ELuc transgenic mouse

The transgene was microinjected into the pronuclei of fertilized zygotes; subsequently, five pups were born (Table 1). Transgenic mice (founder mice) confirmed by genomic PCR were three out of five pups (Table 1). We confirmed that founder mouse No.1 was the brightest (Fig. 2). We decided that founder mouse No.1 was the donor for the generation of the endometriosis animal model.

In vivo and *ex vivo* imaging of the CAG-ELuc transgenic mouse

In founder mouse No.1, ubiquitous expression of the ELuc gene was confirmed by *in vivo* and *ex vivo* imaging (Figs. 3a and 3b). The bioluminescent flux peaked at 5 min after D-luciferin administration, and subsequently decreased. The decay became slow 15 min later (Supplementary Fig. 1).

Detection of the lesion location by *in vivo* imaging and autopsy

It was possible to detect the ELuc signals (Fig. 4a). We confirmed that the signals were derived from the lesion by *ex vivo* imaging (Fig. 4b). On Day 14, the lesions emitting signals were saccate, which is a hallmark of endometriosis-like lesions (Fig. 4c). Before non-invasive and chronological imaging, we verified the precise location of the lesion by autopsy to evaluate the accuracy of lesion location detected by bioluminescence. With both 0.5 and 0.2 μg/mouse of E2 administration, the lesions frequently attached to the areas around the pancreas, suture site, and fatty tissue (Figs. 5a and 5b). Specifically, the fatty tissue was adjacent to the bladder (abdominal part), not the dorsal part. Figure 5c showed whether lesions (or grafts) were detectable by bioluminescence or not. The correspondence rates between the location of bioluminescence and the actual location of lesions or grafts were high, with the lowest value of 57.7% on Day 14 at 0.2 μg/mouse, and the maximum value of 100% on Day3 at 0.5 μg/mouse dose of E2 (Fig. 5c). At 0.2 μg/mouse of E2, the correspondence rates were lower compared to the 0.5 μg/mouse dose on Days 3 and 14 (Fig. 5c). The weight of lesions at the 0.5 μg/mouse dose of E2 was significantly higher compared to the 0.2 μg/mouse dose on Days 3 and 14 (Fig. 5d). The intensity of the luminescence signal was significantly higher at the 0.5 μg/mouse dose of E2 compared to the 0.2 μg/mouse E2 dose on Day 14 but not on Day 3 (Fig. 5e).

Chronological and non-invasive *in vivo* imaging analysis of the endometriosis mouse model

The bioluminescence signals of ELuc could be detected non-invasively over time (Fig. 6). Signals around the pancreas and suture site were detected on Day 1; the signal on the pancreas remained almost at the same position on Day 7; in contrast, the signals around the suture sites decreased.

Discussion

In this study, we established a CAG-ELuc transgenic mouse with powerful bioluminescence expression. Using this transgenic mouse, we generated a novel endometriosis mouse model and traced non-invasively the location of the grafts at a very early phase in endometriosis lesion formation. Interestingly, the location where the grafts were attached was fixed immediately after transplantation of the uterine tissue.

Similar to other models, Becker's group generated a

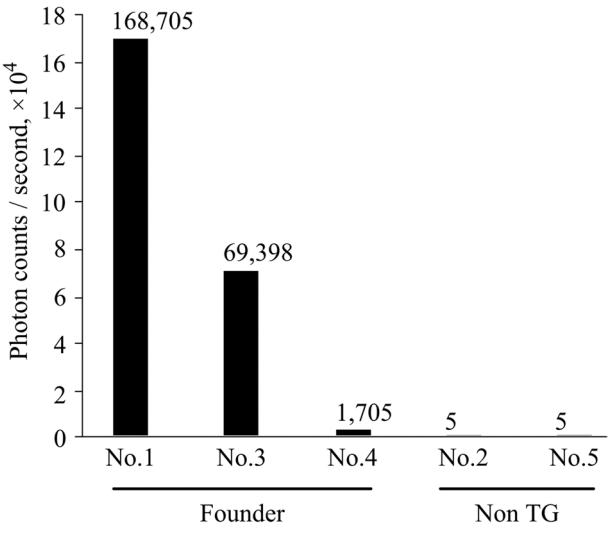

Fig. 2. The generation of CAG-ELuc transgenic mouse. The screening of founders of the CAG-ELuc transgenic mouse by luciferase assay using PHELIOS. The bioluminescence of ELuc was detected with the piece of tails cut from the founders of the CAG-ELuc transgenic mouse. The exposure time was 1 s.

Table 1. The efficiency of generating the CAG-ELuc transgenic mouse

Micro injection	Alive (%)	Two-cell (%)	Embryo transfer	New born	TG (%)
111	97 (87.4)	80 (82.5)	80	5	3 (3.8)

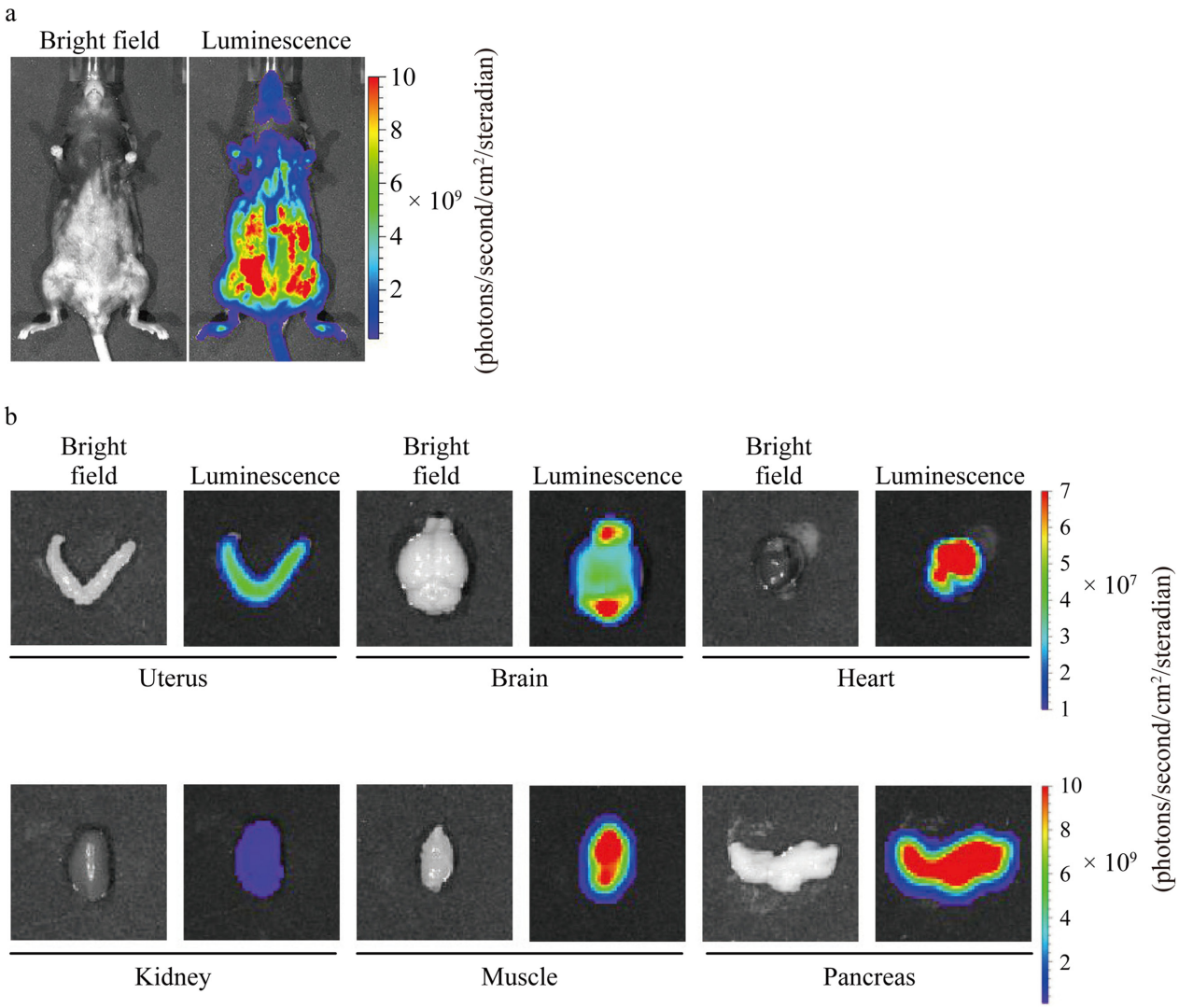

Fig. 3. *In vivo* and *ex vivo* imaging of CAG-ELuc transgenic mouse (founder No. 1). a) The ELuc expression of the whole body was detected by IVIS. The exposure time was 0.2 s. b) The ELuc expression of various organs was detected. The exposure time was 0.2 s.

UbC-Fluc reporter mouse expressing firefly luciferase (Fluc) driven by the ubiquitin C (UbC) promoter [18], and monitored the Fluc-expressing lesion non-invasively for a long time. However, their models needed to create homozygous UbC-Fluc reporter mice, and to set a long exposure time (e.g., 2 min). In our endometriosis animal model using CAG-ELuc mice, ELuc emission could be detected despite mice being hemizygous and a short exposure time (10 s) (Fig. 4a). In addition, our reporter mouse offers an advantage of monitoring even small tissues, e.g., 1 mm × 1 mm or less, at the very early phase (Day 1, Fig. 6).

The precise mechanism by which the grafts attached mostly to the surface of the pancreas (Figs. 5a and 5b) is unclear. The mouse pancreas is positioned in the peritoneal cavity, whereas the human pancreas is located in the retroperitoneum. According to Sampson's hypothesis [2], retrograde menstruation does not reach the pancreas in humans. Indeed, it is rare to find lesions on the pancreas in women with endometriosis. The pancreatic stellate cell identified in the pancreas [19, 20] promotes the release of extracellular matrix components such as collagen by TGF-β1 [21]. Collagen is an extracellular matrix that functions as a ligand of the integrin family for cell-extracellular matrix interactions. Furthermore, the TGF-β family has been extensively studied in endometriosis and is implicated in the development of endometriosis lesions [22]. These may be the clues to explain why grafts easily adhere to the pancreas; we speculated that the pancreas might be a good matrix for graft (tissue) adhesion.

The accuracy of tracing lesions by bioluminescence depended on the E2 concentration. Our data suggested that the optimized dose of E2 concentration is 0.5 μg/

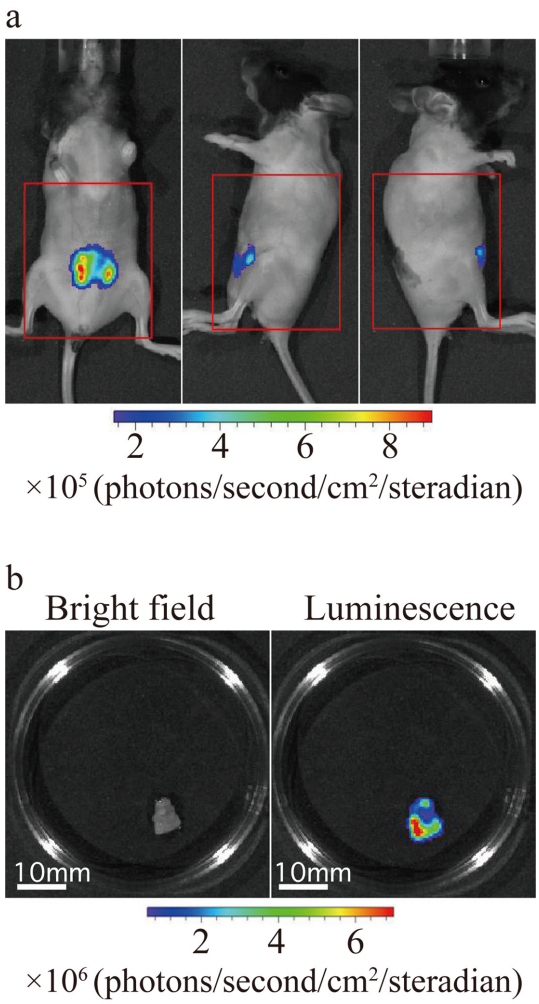

Fig. 4. *In vivo* imaging of endometriosis model using the CAG-ELuc transgenic mouse as the donor. a) An image of the bioluminescence signal in the whole body. The red square indicates ROI. The bioluminescent flux in the ROI square was computed by IVIS. b) *Ex vivo* imaging of the excised lesion. Images on the left and right side are bright field and luminescence, respectively. a, b) The exposure time was 10 s. c) Stereomicroscope image of the endometriosis-like lesion after imaging by IVIS (Day 14).

mouse (Fig. 5c). In estrogen receptor-KO mice, the growth of grafts was reduced [23, 24]. Thus, E2 could be a key factor in lesion development. In the case of E2 (0.5 μg/mouse), it was easy to detect non-invasively the transplanted lesion because larger lesions were detectable (Fig. 5d). The difference in lesion size was not observed by imaging on Day 3, despite being detectable on Day 14 (Figs. 5d and 5e). To evaluate the weight of the lesion by imaging, the weight difference needs to be approximately 50 mg at least (Day 14 in Figs. 5d and 5e).

The grafts preferred specific locations around the pancreas and suture site in the very early phase (Fig. 6, Day 1). On the other hand, the signals except pancreas decreased gradually with time by attenuating the inflammatory state in the peritoneal cavity. Other studies have shown that the engraftment and growth of transferred cells depend on the graft manipulation and recipient condition [25, 26]. Our data suggest that most of the grafts survived Day1 and decreased by necrosis and the action of immune cells as time went by. Simultaneously, angiogenesis increased at the grafts, supporting the development of the graft by providing a sufficient nutrient supply. This may explain why correspondence rates of 0.2 μg/mouse E2 administration in Fig. 5c was lower than 0.5 μg/mouse. E2 enhances expression of vascular endothelial growth factor (VEGF), an angiogenetic marker [27]. Therefore, we speculated that a low nutrient supply due to the insufficient expression of VEGF affected to the viability of grafts, and the bioluminescence signal became weak in 0.2 μg/mouse of E2.

For imaging of the lesion formation, the evaluation of the depth of the lesion in the peritoneal cavity is crucial. The emission intensity is altered by the depth of the lesion. In the experiment using the UbC-Fluc reporter mouse [18], a piece of the uterus was patched on the abdominal peritoneum wall. It is easy to detect luminescence non-invasively despite the weak emission because the organ in the peritoneal cavity does not limit the emission to reach the detector. Therefore, this model is suitable for the quantification of lesion size. In contrast, our model has the advantage of tracing the migration of grafts that can move freely, because even small grafts possess strong emissions. Our model is optimal for tracing grafts in the early phase. A suitable bioluminescence animal and the investigation methodology should be selected to match the study purpose.

In particular, our model could be useful not only for screening therapeutic drugs but also for specific gene expressions critical for endometriosis development. The combination of our model and gene knockout mouse makes it possible to evaluate several factors associated

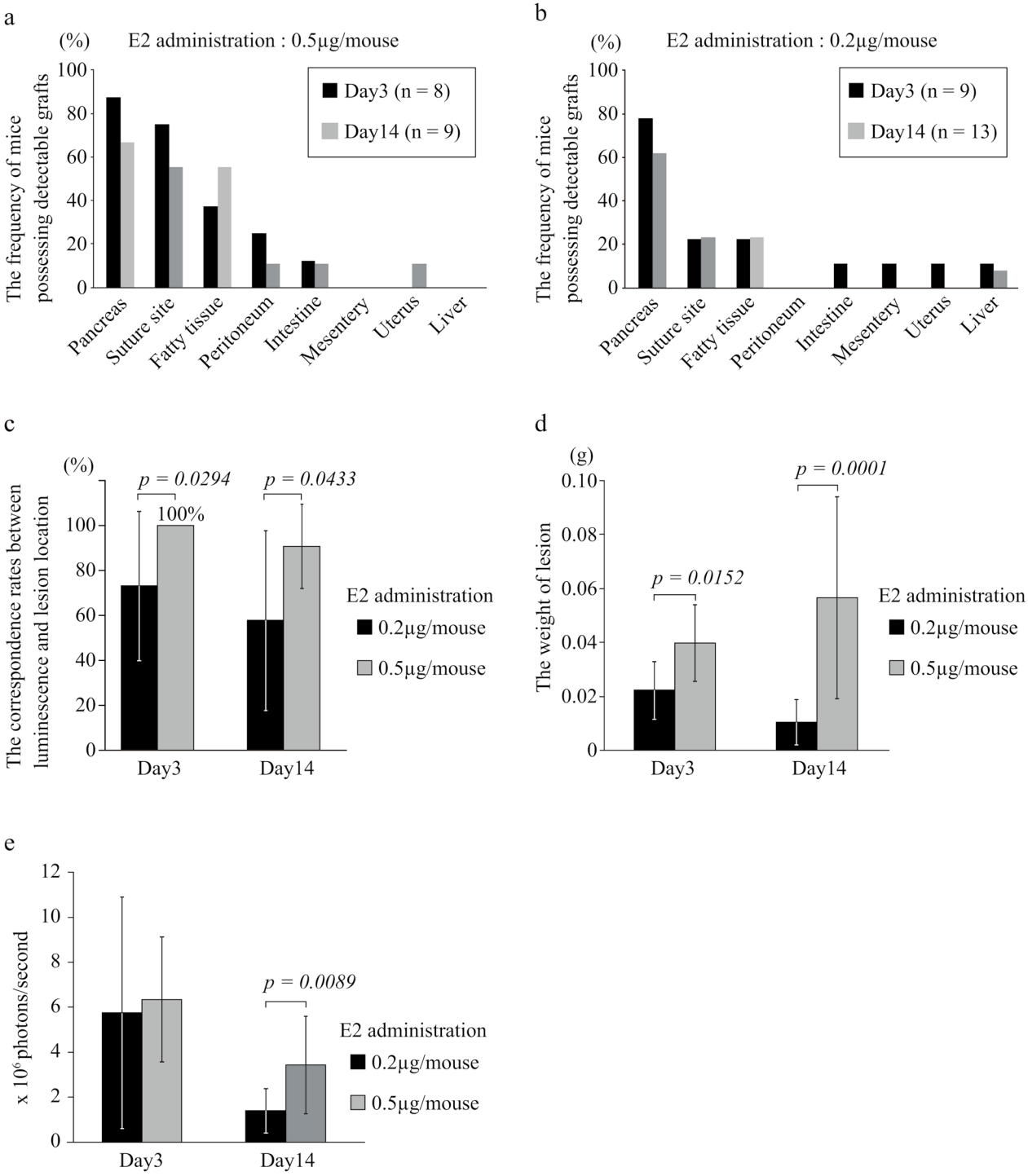

Fig. 5. Detection of the lesion located by *in vivo* imaging and autopsy. a, b) The dose of E2 was 0.5 (a) and 0.2 (b) *μ*g/mouse. The frequency of mice possessing detectable grafts and endometriotic lesions by macroscopic observation is shown as a percentage (mice possessing detectable grafts / all mice). c) The correspondence rates between the location of bioluminescence and the actual location of lesions or grafts confirmed by macroscopic observation. d) The weights of lesions. e) The intensity of non-invasively detected luminescence. The intensity is the mean of three ROIs on the abdominal, right, and left sides (as in Fig. 4a). The exposure time was 10 s.

with the events in the early phase of endometriotic lesion formation. Further, our model contributes to the development of a causal treatment for endometriosis in adolescent women.

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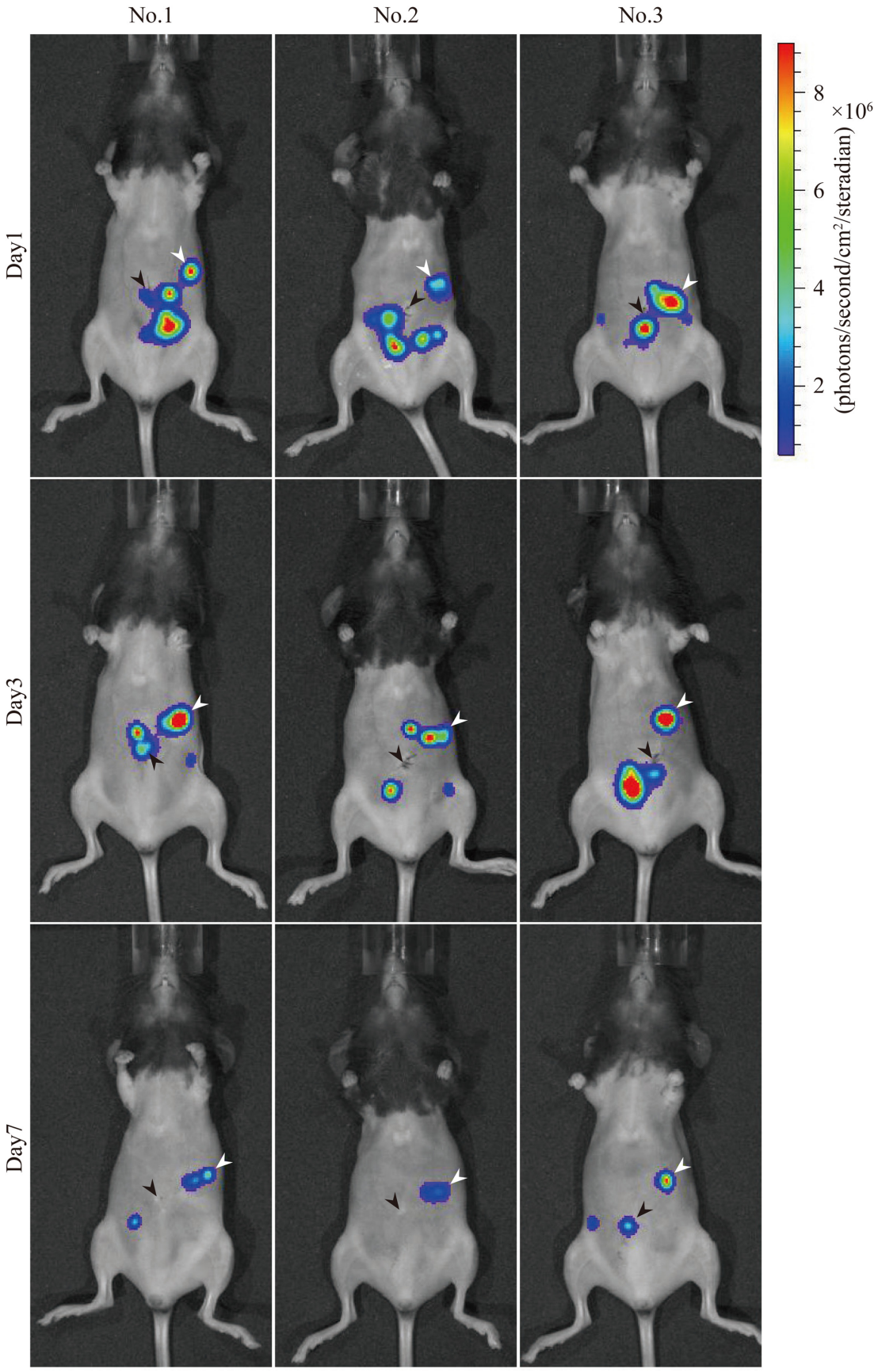

Fig. 6. Chronological and non-invasive *in vivo* imaging analysis of the endometriosis mouse model. The ELuc light emission from the lesion in the peritoneal cavity was detected by IVIS on Days 1, 3, and 7 after transplantation surgery in the same endometriosis model mouse. The exposure time was 10 s. The black and white arrows show suture sites and signals from around the pancreas, respectively.

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