

Review Article

A novel model for endometriosis

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Human uterine endometrium has unique properties to regenerate and remodel cyclically throughout the woman's reproductive life and also gives rise to endometriosis through ectopic implantation of retrograde shedding including endometrial cells during menstruation. Endometriosis is a common and significant gynecological disorder which can lead to infertility or a certain type of ovarian cancer. However, the etiology and pathogenesis still remain uncertain. Previously, we have reported that singly dispersed cells isolated directly from human endometrium can reconstruct the functional ectopic endometria when transplanted beneath the kidney capsule of the NOD/SCID/ γC^{null} immunodeficient mouse. In addition to the endometrium-like structure, hormone-dependent changes (e.g. proliferation, differentiation, tissue breakdown and shedding) characteristic of cycling human endometrium can be reproduced in the endometrial reconstruct whose blood is supplied by human-mouse chimeric vessels. These results indicate that singly dispersed endometrial cells have potential applications for tissue reconstitution, angiogenesis, and human-mouse chimeric vessel formation, providing implications for model mechanisms underlying the establishment of endometriotic lesions and the physiological endometrial regeneration during the menstrual cycle. Furthermore, the hormone-dependent behavior of the endometrium reconstructed from lentivirally-engineered endometrial cells expressing a variant luciferase can be assessed noninvasively and quantitatively by *in vivo* bioluminescence imaging. Our animal model will provide a powerful tool to investigate the pathophysiology of endometriosis and also to validate the effect of novel therapeutic agents and gene targeting on endometriosis with the noninvasive and real-time evaluation system. This animal system can be applied as a unique model for other various types of neoplastic diseases when the relevant cells are transplanted under the kidney capsule. This article describes our novel mouse model and *in vivo* imaging system with overviews of experimental models for endometriosis.

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Introduction

Endometriosis is an estrogen-dependent, chronic gynecological disorder which is characterised by the presence of uterine endometrial tissue outside of the normal location, mainly on the pelvic peritoneum. The prevalence of pelvic endometriosis approaches $\sim 10\%$ in the general female population, and it can cause severe pain and infertility¹⁾. Although it is a benign disease, symptoms recur in up to 75% of women within 2 years after surgery which relieve the pain, and this disorder could increase the risk of ovarian cancer^{1,2)}. However, the etiology and pathogenesis of endometriosis still remain uncertain. One of the biggest problems in this area of research is the lack of appropriate *in vivo* models for endometriosis, because rodents that are most commonly and conveniently used as animal models do not undergo menstruation.

In terms of the most widely accepted developmental mechanism for endometriosis, the “implantation theory”³⁾, retrograde menstruation is essential for peritoneal endometriotic lesions, even if additional factors are needed for establishment and maintenance of endometriosis. In fact, eutopic endometrial cells were deposited into the peritoneal cavity via retrograde menstruation and induced the recurrence of endometriosis⁴⁾. The retrograde menstruation must have “endometriosis initiating cells” which can establish and maintain endometriotic lesions, that is to say the endometriosis initiating cells must originate in human endometrium itself. Because menstrual shedding is a requirement for the development of endometriosis, the animals except for some non-human primates don't have spontaneous endometriosis. However, realistically, it is quite difficult to give experimental evidence on human and non-human primates because of ethical issues, invasive potential of the experiments and high handling costs of non-human primates. Therefore, we believe that the investigation for physiological roles in the pathogenesis of endometriosis is best performed in small animals which have endometriotic lesions of human origin.

Xenotransplanted mouse models

In recent years, immunodeficient mice xenotransplanted with human endometrial tissue fragments have been used as animal models of endometriosis⁵⁾. The survival rate of endometriotic lesions and the time period for maintenance of well-preserved endometriotic tissue are limited by the host immune system, as these factors are shown to improve with increasing immunodeficiency potential of the transplanted mice. Human endometrial tissues have been successfully implanted either subcutaneously or intraperitoneally into several types of immunodeficient mice

including athymic nude mouse⁶⁻¹¹⁾, severe combined immunodeficient (SCID) mice¹²⁻¹⁴⁾, non-obese diabetic (NOD)-SCID mice¹⁰⁾ and NOD/SCID/ γ c^{null} (NOG) mice^{15,16)}. Additionally, menstrual endometrium¹⁷⁻¹⁹⁾ and endometriotic tissue²⁰⁾ have also been engrafted into nude mice and transgenic recombinase-activating gene-2 knockout (RAG-2/ γ (c)KO) mice respectively.

In terms of its capacity to establish and maintain endometriosis, the endometriosis initiating cell could potentially be an endometrial stem/progenitor cell. More recently, transplantation of human endometrial epithelial micro-aggregates (organoids), with dissociated mouse or human endometrial stromal cells, beneath the kidney capsule of nude mice developed human endometrial tissue comprising human glands, mouse or human stroma and myometrium²¹⁾. While this experiment showed the importance of epithelial-stromal interactions, it also indicated the presence of stem/progenitor cells.

Many current models demonstrate sufficient evidence to conclude that rare populations of adult epithelial and stromal stem/progenitor cells exist in human endometrium. However, to study the pathophysiology of endometriosis or the physiology of human endometrial stem cells, the models should meet the following criteria: 1) the transplanted human graft must be quantitatively uniform in each animal, 2) the model should reproduce both functional and morphological changes characteristic of human eutopic and/or ectopic endometrium, and 3) it should be possible to obtain quantitative data of the transplant in real-time, for a prolonged period, without invasive techniques.

Our mouse model for endometriosis

We have recently developed a novel mouse model which fulfills all these requirements²²⁾. When fully dissociated unfractionated human cells (5×10^5) were transplanted directly under the kidney capsule of NOG immunodeficient mice, we observed well-organized tissues including endometrial and myometrial layers in all of the transplanted 60 kidneys of ovariectomized NOG mice which had been hormonally treated with estrogen/progesterone for 10 weeks (Fig.1). It allows relatively easy production of several or even dozens of homogeneous mice from one sample. These ectopic endometria on the kidneys mimicked endometrial hormone-dependent process such as cellular proliferation, differentiation (decidualization) and tissue breakdown (Fig.1A). Furthermore, the transplants after progesterone withdrawal contained a large blood-filled cyst like a red spot lesion of endometriosis (Fig.2) as well as a chocolate cyst generated by subcutaneous grafting of endometriotic lesions into RAG-2/ γ (c)KO mice²⁰⁾. We have demonstrated that singly dispersed human endometrial

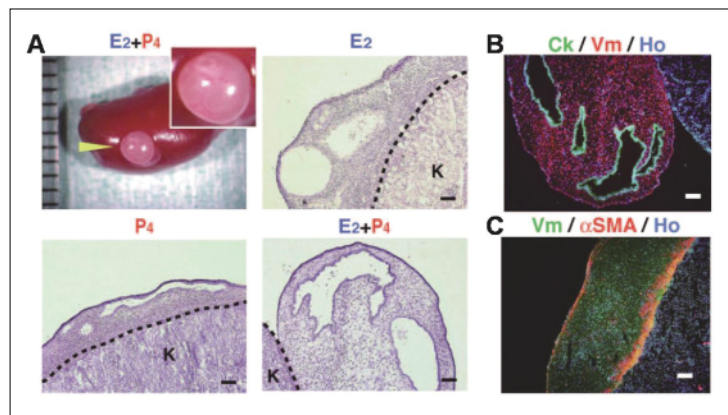


Fig.1 Reconstruction of human endometrium-like tissue from singly dissociated endometrial cells

(A) Macroscopic and microscopic findings of the transplanted site (arrowhead) of NOG mice 10 weeks after xenotransplantation. H&E staining was performed on the transplanted lesion of NOG mice treated with Estoradiol (E2), Progesterone (P4), or E2+P4 as indicated. The borders between the reconstituted tissue and the mouse kidney (K) are indicated by the dotted lines. (B and C) Immunofluorescence staining of the endometrial reconstructs in the E2+P4-treated NOG mice by using antibodies against cytokeratin (Ck) and Vimemtin (Vm) (B), Vm and α -Smooth muscle actine (α SMA) (C), followed by Hoechst (Ho) staining. (Scale bars: 100 μ m) Reproduce from ref. 22 with permission. Copyright 2007, National Academy of Sciences, USA.

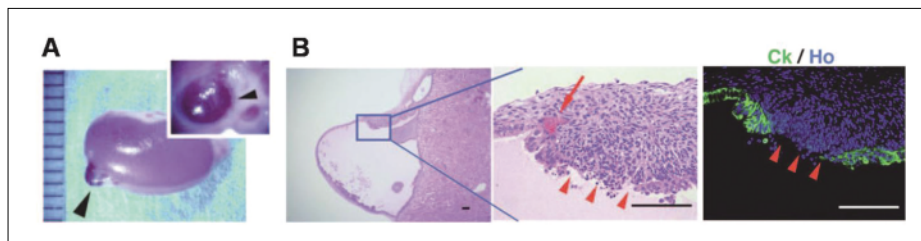


Fig.2 A large blood-filled cyst like a red spot lesion of endometriosis after progesterone withdrawal

(A) Macroscopic findings of the transplanted site (arrows). (B) H&E and immunofluorescence staining of the transplanted lesion. The glandular structure was partially disrupted (arrowheads), and hemorrhage occurred in the stroma (arrow). A small box marks a region shown at higher magnification in the adjacent panel as indicated. (Scale bars: 100 μ m)

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cells can reconstitute ectopic functional endometrial tissues, mimicking human endometriosis²²).

Unique angiogenic potential of human endometrial cells

While host angiogenesis into the human transplants was observed as described previously^{10,17,19,22-24}), our results showed that human blood vessels invaded into the murine kidney parenchyma and formed chimeric vessels with the host murine endothelium,

providing a murine blood flow to the transplant (Fig.3). Previously, it has been reported that human graft vessels disappeared gradually in other endometriosis models^{10,19,23,24}). Although tumor-derived endothelial cells are known to demonstrate an angiogenic ability to grow into immunodeficient mice and formed vasculatures connected with the host circulation²⁵), this is the first report of the angiogenesis from human transplants derived from normal, not cancer, cells into the host tissue²²). A lack of NK cell activity in NOG mice might allow us to see the unique ability of

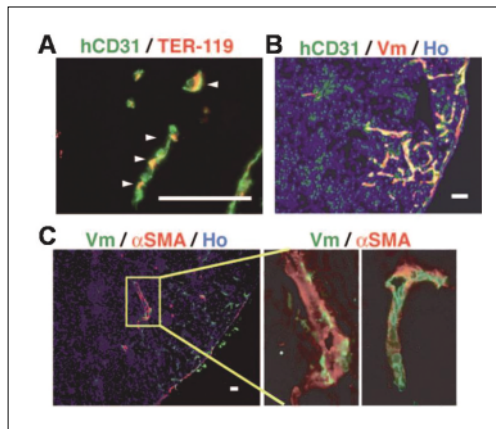


Fig.3 Vascularization of the reconstructed endometrium

Immunofluorescence staining of the reconstituted endometrial tissue (A) and the mouse kidney parenchyma adjacent to the reconstituted tissue (B and C) by using antibodies against human CD31 (hCD31) and TER-119 (A), hCD31 and Vm (B), or Vm and αSMA (C), followed by Hoechst (Ho) staining. (C) A small box marks a region shown at higher magnification in the adjacent panel as indicated. (Scale bars: 100 μm)

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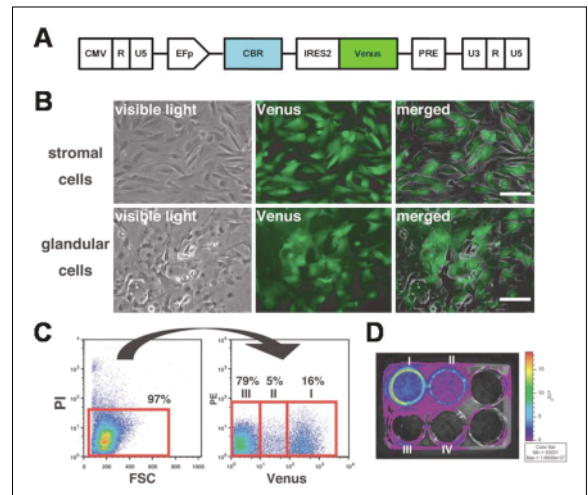


Fig.4 Lentiviral construct for fluorescence and bioluminescence and expression of both fluorescent and luminescent markers in the lentivirally transduced endometrial cells

(A) Lentiviral construct encoding a dual function CBR luc (a luciferase variant) and Venus (a YFP variant) bicistronic reporter gene connected via an internal ribosomal entry site (IRES). (B) Phase-contrast and fluorescence microscopy of the primary cultures of lentivirally transduced endometrial stromal (Upper) and glandular (Lower) cells. (C) A representative flow cytometric profile of the PI negative fraction (red box at Left) of the lentivirally transduced endometrial cells consisting of three subpopulations (Right) based on the fluorescent intensity: high (I), low (II), and negative (III) subpopulations. (D) Macroscopic luminescent image of a six-well dish where each subpopulation, as sorted in B, was cultured in the corresponding well. Non-infected endometrial cells were cultured in the IV well.

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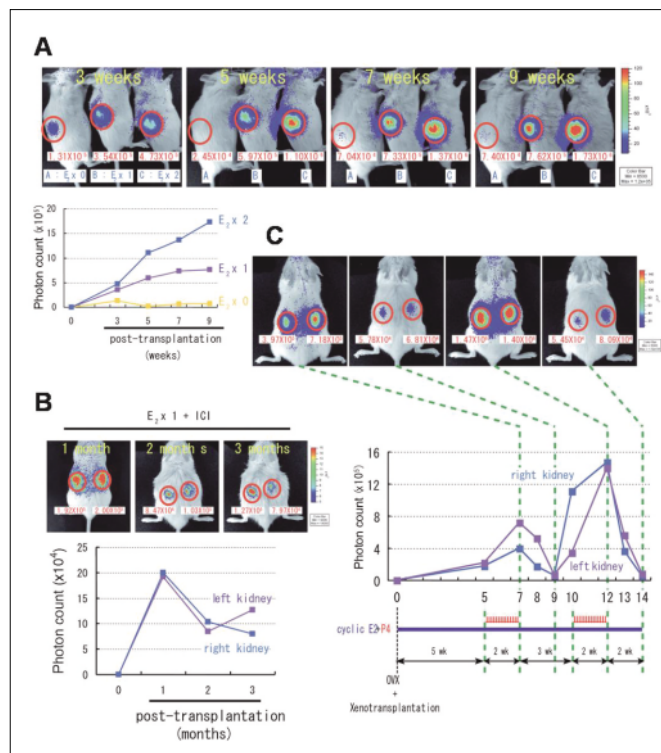


Fig.5 Quantitative assessment of the growth of the reconstructed endometria under the kidney capsules of ovariectomized NOG mice

Representative BLI (top panels) and serial photon count measurements (second panel) of NOG mice treated for different durations with the various indicated doses of E2 pellets (A), with E2 in combination with daily injections of ICI 182,780, a pure estrogen antagonist (B), or with cyclic E2+P4 treatment (bottom panel) to induce artificial menstrual cycle-related changes (C). The photon count value of each region of interest (ROI, red circle) is indicated.

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endometrial epithelial cells because NK cells play a critical role in the IL-12-mediated inhibition of angiogenesis²⁶. Importantly, our observation newly suggests that human endothelial cells/progenitors in endometrium can migrate, invade, form chimeric vasculature in host tissue, even that of a different species, and establish the functional circulatory system. Either way, since the functional vascularization is absolutely required for maintenance of endometriotic lesion, the angiogenic potential of the endometrial endothelial cells itself may be crucial for establishment and development endometriosis. Anti-angiogenic therapies have been recently proposed as a potential alternative treatment for endometriosis^{24,27,28}. This therapeutic strategy might be further strengthened by targeting endometrium or endometriosis specific angiogenesis.

Monitoring system for our model

Single-cells transplantation is adequate for several experimental procedures such as cell selection, genetic engineering, and quantitative assessment before transplantation. In our study, we selected the lentivirally infected cells via their flouorochrome by flow cytometry and transplanted a certain number of these infected cells into each kidney (Fig.4). Then, we detected the bioluminescence emitted from living transplanted cells by Bioluminescence Imaging (BLI) technique and obtain the quantitative data of the reconstituted tissues in real-time, for a prolonged period, without invasive techniques (Fig.5). To achieve non-invasive detection and quantification of transplants, dissociated glandular and stromal cells labelled with lipophylic dye (DiO)²⁹ and transduced endometrial fragments with GFP cDNA³⁰ were subcutaneously or intraperitoneally transplanted into nude mice. However, transplanted cells and tissues scattered or spread unexpectedly, the emitted lights were diluted with cell division and the permeability of the light was limited. Therefore, we used the lentivirus³¹ capable of introducing and stably expressing the coding gene and both Venus (a YFP variant)³² and click beetle red-emitting (CBR) luciferase were encoded on the lentivirus as reporter genes (Fig.4A). Venus was useful for flow cytometric selection of lentivirally infected cells (Fig.4B). CBR emitting light has the capacity to pass through thicker tissues and this enabled us to detect the viable cells from outside the murine body by *in vivo* BLI²². Additionally, kidney capsule transplantation allowed a small number of the cells to be localized and grow three-dimensionally at the relatively superficial location. Combining our model together with advantages of the Bioluminescence Imaging (BLI) technique³³, Firstly, we demonstrated that the signal intensities reflecting the volume of the recon-

structed tissue were enhanced in an estrogen dose- and time-dependent manner (Fig.5A). Therefore, the growth behaviour of the reconstructed tissue was assessed quantitatively and sequentially. Meanwhile, the signal intensity was not increased but rather decreased 2-3 months after co-treatment with ICI-182,780 (ICI), a pure estrogen antagonist³⁴ (Fig.5B). These data indicated that the antagonistic effect of ICI can be noninvasively and successfully assessed and this system could be used as a tool for drug screening. Finally, we could monitor successfully the menstrual cycle-related dynamic changes of the transplants for an extended period in a noninvasive, real-time, and quantitative manner (Fig.5C). Sequential BLI revealed that the signal intensities fluctuated dramatically in accord with the addition and withdrawal of progesterone (Fig.5C). In particular, tissue breakdown and regression after progesterone withdrawal and subsequent tissue regeneration faithfully reflected the decrease and increase in signal intensity, respectively.

Conclusive Remarks

Our animal system showed that singly dissociated human endometrial cells produce the ectopic endometrium. This model indicated that singly dissociated human endometrial cells had endometriosis initiating cells and endometrial stem /progenitor cells. Simultaneously, we proposed the unique animal model is suitable to study the pathogenesis of endometriosis through noninvasive, real-time, and quantitative assessment of ectopically reconstituted endometrium-like tissues. This novel animal model system can also provide opportunities of drug testing and gene-target validation in endometriosis²². Furthermore it would be potentially applicable for other various types of neoplastic diseases when the relevant primary culture cells or cell lines are transplanted beneath the kidney capsule.

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References

- Giudice LC, Kao LC: Endometriosis. *Lancet*, 364: 1789-1799, 2007.
- Bulun SE: Endometriosis. *N Engl J Med*, 360: 268-279, 2009.
- Sampson JA: Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. *The Am J Pathol*, 3: 93-110.43, 1927.
- Bulletti C, DeZiegler D, Stefanetti M, Cicinelli E, Pelosi E,

- Flamigni C: Endometriosis: absence of recurrence in patients after endometrial ablation. *Hum Reprod*, 16: 2676-2679, 2001.
- 5) Grummer R: Animal models in endometriosis research. *Hum Reprod Update*, 12: 641-649, 2006.
 - 6) Zamah NM, Dodson MG, Stephens LC, Buttram VC, Jr., Besch PK, Kaufman RH: Transplantation of normal and ectopic human endometrial tissue into athymic nude mice. *Am J Obstet Gynecol*, 149: 591-597, 1984.
 - 7) Bergqvist A, Jeppsson S, Kullander S, Ljungberg O: Human uterine endometrium and endometriotic tissue transplanted into nude mice. Morphologic effects of various steroid hormones. *Am J Pathol*, 121: 337-341, 1985.
 - 8) Zaino RJ, Satyaswaroop PG, Mortel R: Histologic response of normal human endometrium to steroid hormones in athymic mice. *Hum Pathol*, 16: 867-872, 1985.
 - 9) Bruner KL, Matrisian LM, Rodgers WH, Gorstein F, Osteen KG: Suppression of matrix metalloproteinases inhibits establishment of ectopic lesions by human endometrium in nude mice. *J Clin Invest*, 99: 2851-2857, 1997.
 - 10) Grummer R, Schwarzer F, Bainczyk K, et al: Peritoneal endometriosis: validation of an in-vivo model. *Hum Reprod*, 16: 1736-1743, 2001.
 - 11) Beliard A, Noel A, Goffin F, Frankenne F, Foidart JM: Role of endocrine status and cell type in adhesion of human endometrial cells to the peritoneum in nude mice. *Fertil Steril*, 78: 973-978, 2002.
 - 12) Aoki D, Katsuki Y, Shimizu A, Kakinuma C, Nozawa S: Successful heterotransplantation of human endometrium in SCID mice. *Obstet Gynecol*, 83: 220-228, 1994.
 - 13) Kaufmann R, Rudolphi A, Boxberger HJ, Hainzl A, Rosenthal H, Reimann J: Stable engraftment of human female genital mucous membrane xenografts on SCID mice. *Gynecol Obstet Invest*, 40: 97-100, 1995.
 - 14) Awwad JT, Sayegh RA, Tao XJ, Hassan T, Awwad ST, Isaacson K: The SCID mouse: an experimental model for endometriosis. *Hum Reprod*, 14: 3107-3111, 1999.
 - 15) Ito M, Hiramatsu H, Kobayashi K, et al: NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. *Blood*, 100: 3175-3182, 2002.
 - 16) Matsuura-Sawada R, Murakami T, Ozawa Y, et al: Reproduction of menstrual changes in transplanted human endometrial tissue in immunodeficient mice. *Hum Reprod*, 20: 1477-1484, 2005.
 - 17) Nisolle M, Casanas-Roux F, Donnez J: Early-stage endometriosis: adhesion and growth of human menstrual endometrium in nude mice. *Fertil Steril*, 74: 306-312, 2000.
 - 18) Van Langendonck A, Casanas-Roux F, Eggermont J, Donnez J: Characterization of iron deposition in endometriotic lesions induced in the nude mouse model. *Hum Reprod*, 19: 1265-1271, 2004.
 - 19) Eggermont J, Donnez J, Casanas-Roux F, Scholtes H, Van Langendonck A: Time course of pelvic endometriotic lesion revascularization in a nude mouse model. *Fertil Steril*, 84: 492-499, 2005.
 - 20) Greenberg LH, Slayden OD: Human endometriotic xenografts in immunodeficient RAG-2/gamma(c)KO mice. *Am J Obstet Gynecol*, 190: 1788-1795; discussion 95-96, 2004.
 - 21) Kurita T, Medina R, Schabel AB, et al: The activation function-1 domain of estrogen receptor alpha in uterine stromal cells is required for mouse but not human uterine epithelial response to estrogen. *Differentiation*, 73: 313-322, 2005.
 - 22) Masuda H, Maruyama T, Hiratsu E, et al: Noninvasive and real-time assessment of reconstructed functional human endometrium in NOD/SCID/gamma c(null) immunodeficient mice. *Proc Natl Acad Sci USA*, 104: 1925-1930, 2007.
 - 23) Bruner-Tran KL, Webster-Clair D, Osteen KG: Experimental endometriosis: the nude mouse as a xenographic host. *Ann N Y Acad Sci*, 955: 328-339; discussion 40-42, 396-406, 2002.
 - 24) Hull ML, Charnock-Jones DS, Chan CL, et al: Antiangiogenic agents are effective inhibitors of endometriosis. *J Clin Endocrinol Metab*, 88: 2889-2899, 2003.
 - 25) Bussolati B, Deambrosis I, Russo S, Deregibus MC, Camussi G: Altered angiogenesis and survival in human tumor-derived endothelial cells. *Faseb J*, 17: 1159-1161, 2003.
 - 26) Yao L, Sgadari C, Furuke K, Bloom ET, Teruya-Feldstein J, Tosato G: Contribution of natural killer cells to inhibition of angiogenesis by interleukin-12. *Blood*, 93: 1612-1621, 1999.
 - 27) Dabrosin C, Gyorffy S, Margetts P, Ross C, Gauldie J: Therapeutic effect of angiostatin gene transfer in a murine model of endometriosis. *Am J Pathol*, 161: 909-918, 2002.
 - 28) Nap AW, Griffioen AW, Dunselman GA, et al: Antiangiogenesis therapy for endometriosis. *J Clin Endocrinol Metab*, 89: 1089-1095, 2004.
 - 29) Tabibzadeh S, Miller S, Dodson WC, Satyaswaroop PG: An experimental model for the endometriosis in athymic mice. *Front Biosci*, 4: C4-C9, 1999.
 - 30) Fortin M, Lepine M, Page M, et al: An improved mouse

model for endometriosis allows noninvasive assessment of lesion implantation and development. *Fertil Steril*, 80(Suppl 2): 832-838, 2003.

- 31) Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM: Development of a self-inactivating lentivirus vector. *J Virol*, 72: 8150-8157, 1998.
- 32) Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A: A variant of yellow fluorescent protein with

fast and efficient maturation for cell-biological applications. *Nat Biotechnol*, 20: 87-90, 2002.

- 33) Masuda H, Okano HJ, Maruyama T, Yoshimura Y, Okano H, Matsuzaki Y: In vivo imaging in humanized mice. *Curr Top Microbiol Immunol*, 324: 179-196, 2008.
- 34) Wakeling AE, Dukes M, Bowler J: A potent specific pure antiestrogen with clinical potential. *Cancer Res*, 51: 3867-3873, 1991.