with platelet-derived growth factor (PDGF), are mitogens for epithelial cells from the basal layer [87]. The best evidence of early endometrial growth is presented in a scanning electron microscopic study of human endometrium by Ludwig and Spornitz [88]. After the shedding of the functional layer, the exposed surface is covered by fibronectin and leukocytes. This fibronectin is rapidly removed once epithelialization occurs. Regrowth of the epithelium, beginning from the stumps of the glands, starts on menstrual day 2. The surface epithelium grows out of the cone-shaped gland edges, rapidly covering the luminal surface, two thirds of which is covered by day 4. By day 6, epithelialization is complete [89].

Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing vasculature. Regularly repeated, programmed vessel growth and remodeling, which rarely take place in the normal adult outside of the female reproductive tract, is required for the cyclic processes of endometrial shedding and regeneration. There are four phases of the endometrial cycle accompanied by important angiogenic events: repair of ruptured blood vessels at menstruation, vessel elongation during the proliferative phase, development of the spiral arterioles during the secretory phase, and vascular regression in the premenstrual phase [90]. Impairment of proper angiogenic remodeling may result in abnormalities of endometrial function including menorrhagia.

Endometrial angiogenesis and vessel remodeling are driven by a network of signaling molecules and receptors that include members of the vascular endothelial growth factor (VEGF) family, their splicing variants, fibroblast growth factors, angiopoietins, angiogenin, and the ephrins and their cognate receptors [91]. Members of VEGF-A are fundamental to endometrial angiogenesis, in particular, during the peri- and post-menstrual periods. VEGF-A levels are highest in the menstrual phase, presumably in response to pro-inflammatory cytokines. This temporal, prominent expression might also be attributable to focal hypoxia partly resulting from vasoconstriction, which potently stimulates VEGF-A gene transcription. Expression of two different receptors for VEGF-A members, VEGFR1 and VEGFR2, are also most prominent in the menstrual phase. Thus, the increased levels of VEGF and cognate receptor expression in the menstrual phase are presumed to be prerequisites for vessel repair and the preparation for angiogenesis in the proliferative phase [92, 93]. In addition to VEGF members, several other angiogenic factors and their cognate receptors are temporally and spatially expressed in the endometrium [91]. The specific roles of each of these factors in the endometrial angiogenesis-vessel remodeling cycle, however, remain to be elucidated.

Stem cells

The human endometrium exhibits a tremendous regenerative capacity that enables cyclical regeneration and remodeling throughout a woman's reproductive life. Indeed, each month, the endometrial mucosa grows approximately 1 cm in about 10–14 days, equivalent to the level of tissue regeneration occurring in other high-turnover tissues, such as the skin, gastrointestinal tract, and bone marrow [94]. Such regeneration and remodeling in the endometrium allude to the existence of endometrial stem and progenitor cells. These cells are postulated to reside in the basalis layer, which is retained during menstruation [95]. Recently, several candidate populations of adult stem/progenitor cells in not only the human endometrium but also the human uterine myometrium have been identified [96–99].

A novel experimental model for endometrial regeneration and angiogenesis

The research on endometrial regeneration and angiogenesis is complicated by major species differences between the menstrual cycle in humans and primates and the estrus cycle in commonly studied rodent models. Although rodent models provide invaluable information, caution is required when translating information to the human menstrual cycle. Despite the difficulties, the endometrium is an important model for studying physiological angiogenesis in adults well as angiogenesis in pathological conditions such as endometriosis.

To study the physiology of the human endometrium and the pathogenesis of endometriosis, a variety of *in vivo* animal models have been developed [100]. The current *in vivo* models, however, do not completely satisfy the following requirements: (i) the transplanted human tissue must be quantitatively and characteristically uniform in each animal, (ii) functional and mor-

phological changes characteristic of human eutopic and/or ectopic endometrium should be reproduced, and (iii) the transplant needs to be assessable for an extended period with noninvasive, real-time, and quantitative measures.

We have recently developed a novel mouse model that meets all of these requirements [101]. In brief, human endometrial specimens, collected from consenting patients with benign gynecological diseases, were mechanically and enzymatically dissociated into singly dispersed endometrial cells (SDECs). We transplanted SDECs beneath the kidney capsules of severely immunodeficient NOD/SCID/γ_c^{null} (NOG) mice. The NOG mice possess multiple immunological deficiencies, including cytokine production incapacity and functional incompetence of T, B, and natural killer cells. These mice are, therefore, ideal candidates for receiving xenografts and have high rates of graft acceptance [102]. At transplantation, recipient NOG mice underwent ovariectomy to eliminate the influence of endogenous ovarian steroid hormones. They were then treated without or with E_2 alone or in combination with progesterone for several weeks. Some xenotransplanted NOG mice were subjected to cyclical hormonal treatment to reproduce an artificial menstrual cycle. Histological and immunofluorescence analyses were performed on the endometrium-like reconstructions that grew under the kidney capsules. We found that endometrium-like tissues with tissue polarity, glandular structures, and endometrial cell components could be regenerated from SDECs in all the xenotransplanted NOG mice (n = 30)(Fig. 3A). In addition to duplicating an endometrium-like structure, the transplanted tissue exhibited hormone-dependent changes including proliferation, differentiation, tissue breakdown, and shedding (menstruation) (Fig. 3B). In the endometrial reconstructs, there existed chimeric vessels comprised of human and mouse vessels that functioned as a circulatory system (Fig.3, C and D).

Bioluminescence imaging (BLI) recently has emerged as a useful tool for tumor, hematopoietic, and neural cell tracking studies in living animals [103, 104]. We assessed the dynamic state of the endometrial reconstructs derived from the genetically engineered SDECs by *in vivo* BLI [101]. For this purpose, SDECs were infected with a lentivirus expressing a variant luciferase reporter gene prior to transplantation beneath the kidney capsule, on the dorsal side, in ovariectomized NOG mice. *In vivo* BLI revealed that the growth pattern of the reconstructed tissue derived from lentiviral-engineered cells could be assessed noninvasively, quantitatively, and sequentially, as determined by the magnitude of photon counts generated by the luciferase reaction [101]. This system enabled the dynamic changes of the endometrial reconstructs, occurring during an artificial menstrual cycle induced by cyclic hormonal treatment, to be monitored noninvasively (Fig. 3E).

Thus, we demonstrated that SDECs have the capacity for tissue regeneration and reconstruction with neovascularization. This regeneration potential indicates that the dissociated endometrial cells, resulting from menstrual breakdown, may participate in the establishment of endometriosis. Angiogenesis likely plays a critical role in the establishment of ectopic endometrial explants derived from retrograde menstruation. Combining the unique potential of SDECs together with NOG mice and lentivirus-mediated cell engineering, we present a novel animal model suitable for the study of endometrial physiology/pathophysiology. With this model, the pathogenesis of endometriosis may be investigated through the noninvasive, real-time, and quantitative assessment of ectopically reconstituted endometrium-like tissues. Furthermore, this animal model system, in which cells engineered with a lentivirus to express a bioluminescence marker are transplanted beneath the kidney capsule, has potential applications for drug testing and gene target validation not only in endometrium-derived disorders, but also in various other types of neoplastic disease.

Concluding remarks

The human endometrium is unique in that it exhibits a tremendous regenerative capacity that enables cyclical regeneration, differentiation, and remodeling throughout a woman's reproductive life. Progesterone together with cAMP is the primary driving force for differentiation. SRC signaling also plays a prominent role in decidualization. The regenerative capacity of endometrial cells makes them ideal candidates for tissue reconstitution, angiogenesis, and human-mouse chimeric vessel formation. Our recently developed, "humanized", mouse model of the reconstituted functional human endometrium may be used to study endometrial differentiation, angiogenesis, and regeneration *in vivo* and improve the understanding of

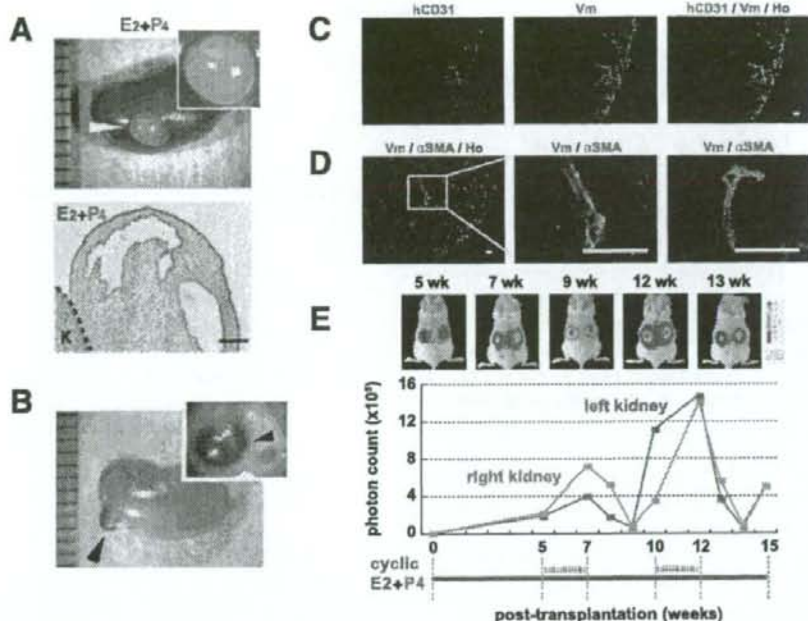

Fig. 3. Regeneration model of human endometrium in mice

A. Macroscopic and microscopic findings of the transplant site (arrowhead) in NOG mice 10 weeks after xenotransplantation. H&E staining was performed on the transplanted lesion of NOG mice treated with E_2 in combination with progesterone (E_2+P_4). The borders between the reconstituted tissue and the mouse kidney (K) are indicated by the dotted lines. Bar, 100 μm.

B. Macroscopic findings of the transplanted site (arrows) of a NOG mouse after cyclic E_2+P_4 treatment.

C and D. Immunofluorescence staining of the mouse kidney parenchyma adjacent to the reconstituted tissue in the E_2+P_4-treated NOG mice using antibodies against human CD31 (hCD31) and human vimentin (Vm) (C) or Vm and α-smooth muscle actin (αSMA) (D). Bars, 100 μm.

E. Optical bioluminescence images and the noninvasive quantitative assessment of the endometrial tissues reconstructed from lentiviral-transduced SDECs in living NOG mice. Representative BLI (*Upper*) and serial photon count measurements (*Lower*) of xenotransplanted and ovariectomized NOG mice treated with cyclic E_2+P_4 treatment to induce artificial menstrual cycle-related changes. (Ref.101. Reproduced with permission from Masuda *et al.*: Proc. Natl. Acad. Sic. USA, 104: 1925–1930. 2007).

endometrial physiology and pathophysiology. Our model may also be used to identify and test new therapeutic strategies for endometriosis, endometrial cancer, implantation failure, and infertility related to endometrial dysfunction.

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References

1. Gurpide E, Tabanelli S, Tang B (1992) Human endometrial stromal cells. In: Genazzani, A.R. and Petraglia, F. (eds) Hormones in Gynecological Endocrinology. Partenon Press, Casterton Hall. Carnforth, Lancs, UK: 717–724.
2. Tabibzadeh S (1991) Human endometrium: an active site of cytokine production and action. *Endocr Rev* 12: 272–290.
3. Giudice LC (1994) Growth factors and growth modulators in human uterine endometrium: their potential relevance to reproductive medicine. *Fertil Steril* 61: 1–17.
4. Maruyama T, Kitaoka Y, Sachi Y, Nakanoin K, Hirota K, Shiozawa T, Yoshimura Y, Fujii S, Yodoi J (1997) Thioredoxin expression in the human endometrium during the menstrual cycle. *Mol Hum Reprod* 3: 989–993.
5. Maruyama T, Sachi Y, Furuke K, Kitaoka Y, Kanzaki H, Yoshimura Y, Yodoi J (1999) Induction of thioredoxin, a redox-active protein, by ovarian steroid hormones during growth and differentiation of endometrial stromal cells *in vitro*. *Endocrinology* 140: 365–372.
6. Dimitriadis E, White CA, Jones RL, Salamonsen LA (2005) Cytokines, chemokines and growth factors in endometrium related to implantation. *Hum Reprod Update* 11: 613–630.
7. Dunn CL, Kelly RW, Critchley HO (2003) Decidualization of the human endometrial stromal cell: an enigmatic transformation. *Reprod Biomed Online* 7: 151–161.
8. Gellersen B, Brosens J (2003) Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair. *J Endocrinol* 178: 357–372.
9. Tsai MJ, O'Malley BW (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 63: 451–486.
10. Gao J, Mazella J, Tseng L (2002) Hox proteins activate the IGFBP-1 promoter and suppress the function of hPR in human endometrial cells. *DNA Cell Biol* 21: 819–825.
11. McKenna NJ, O'Malley BW (2002) Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* 108: 465–474.
12. Li X, O'Malley BW (2003) Unfolding the action of progesterone receptors. *J Biol Chem* 278: 39261–39264.
13. Brosens JJ, Hayashi N, White JO (1999) Progesterone receptor regulates decidual prolactin expression in differentiating human endometrial stromal cells. *Endocrinology* 140: 4809–4820.
14. Mote PA, Balleine RL, McGowan EM, Clarke CL (1999) Colocalization of progesterone receptors A and B by dual immunofluorescent histochemistry in human endometrium during the menstrual cycle. *J Clin Endocrinol Metab* 84: 2963–2971.
15. Mulac-Jericevic B, Conneely OM (2004) Reproductive tissue selective actions of progesterone receptors. *Reproduction* 128: 139–146.
16. Richer JK, Lange CA, Manning NG, Owen G, Powell R, Horwitz KB (1998) Convergence of progesterone with growth factor and cytokine signaling in breast cancer. Progesterone receptors regulate signal transducers and activators of transcription expression and activity. *J Biol Chem* 273: 31317–31326.
17. Christian M, Pohnke Y, Kempf R, Gellersen B, Brosens JJ (2002) Functional association of PR and CCAAT/enhancer-binding protein beta isoforms: promoter-dependent cooperation between PR-B and liver-enriched inhibitory protein, or liver-enriched activatory protein and PR-A in human endometrial stromal cells. *Mol Endocrinol* 16: 141–154.
18. Kim JJ, Buzzio OL, Li S, Lu Z (2005) Role of FOXO1A in the regulation of insulin-like growth factor-binding protein-1 in human endometrial cells: interaction with progesterone receptor. *Biol Reprod* 73: 833–839.
19. Wehling M (1997) Specific, nongenomic actions of steroid hormones. *Annu Rev Physiol* 59: 365–393.
20. Zhu Y, Bond J, Thomas P (2003) Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. *Proc Natl Acad Sci USA* 100: 2237–2242.
21. Zhu Y, Rice CD, Pang Y, Pace M, Thomas P (2003) Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc Natl Acad Sci USA* 100: 2231–2236.
22. Fernandes MS, Pierron V, Michalovich D, Astle S, Thornton S, Peltoketo H, Lam EW, Gellersen B, Huhtaniemi I, Allen J, Brosens JJ (2005) Regulated expression of putative membrane progestin receptor homologues in human endometrium and gestational tissues. *J Endocrinol* 187: 89–101.
23. Boonyaratanakornkit V, Scott MP, Ribon V, Sherman L, Anderson SM, Maller JL, Miller WT, Edwards DP (2001) Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. *Mol Cell* 8: 269–280.
24. Pohnke Y, Kempf R, Gellersen B (1999) CCAAT/enhancer-binding proteins are mediators in the protein kinase A-dependent activation of the decidual prolactin promoter. *J Biol Chem* 274: 24808–24818.
25. Sterneck E, Tessarollo L, Johnson PF (1997) An essential role for C/EBPbeta in female reproduction. *Genes*

Dev 11: 2153–2162.
26. Robinson GW, Johnson PF, Hennighausen L, Sterneck E (1998) The C/EBPbeta transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. *Genes Dev* 12: 1907–1916.
27. Seagroves TN, Krnacik S, Raught B, Gay J, Burgess-Beusse B, Darlington GJ, Rosen JM (1998) C/EBPbeta, but not C/EBPalpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. *Genes Dev* 12: 1917–1928.
28. Descombes P, Schibler U (1991) A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* 67: 569–579.
29. Christian M, Zhang X, Schneider-Merck T, Unterman TG, Gellersen B, White JO, Brosens JJ (2002) Cyclic AMP-induced forkhead transcription factor, FKHR, cooperates with CCAAT/enhancer-binding protein beta in differentiating human endometrial stromal cells. *J Biol Chem* 277: 20825–20832.
30. Ghosh AK, Lacson R, Liu P, Cichy SB, Danilkovich A, Guo S, Unterman TG (2001) A nucleoprotein complex containing CCAAT/enhancer-binding protein beta interacts with an insulin response sequence in the insulin-like growth factor-binding protein-1 gene and contributes to insulin-regulated gene expression. *J Biol Chem* 276: 8507–8515.
31. Huang H, Tindall DJ (2007) Dynamic FoxO transcription factors. *J Cell Sci* 120: 2479–2487.
32. Kim JJ, Taylor HS, Akbas GE, Foucher I, Trembleau A, Jaffe RC, Fazleabas AT, Unterman TG (2003) Regulation of insulin-like growth factor binding protein-1 promoter activity by FKHR and HOXA10 in primate endometrial cells. *Biol Reprod* 68: 24–30.
33. Schindler C, Darnell JE, Jr. (1995) Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu Rev Biochem* 64: 621–651.
34. Jabbour HN, Critchley HO, Boddy SC (1998) Expression of functional prolactin receptors in nonpregnant human endometrium: janus kinase-2, signal transducer and activator of transcription-1 (STAT1), and STAT5 proteins are phosphorylated after stimulation with prolactin. *J Clin Endocrinol Metab* 83: 2545–2553.
35. Mak IY, Brosens JJ, Christian M, Hills FA, Chamley L, Regan L, White JO (2002) Regulated expression of signal transducer and activator of transcription, Stat5, and its enhancement of PRL expression in human endometrial stromal cells *in vitro*. *J Clin Endocrinol Metab* 87: 2581–2588.
36. Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* 365: 855–859.
37. Janknecht R, Hunter T (1996) Versatile molecular glue. Transcriptional control. *Curr Biol* 6: 951–954.
38. Kamei Y, Xu L, Heinzel T, Torchia J, Kurokawa R, Gloss B, Lin SC, Heyman RA, Rose DW, Glass CK, Rosenfeld MG (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85: 403–414.
39. Mink S, Haenig B, Klempnauer KH (1997) Interaction and functional collaboration of p300 and C/EBPbeta. *Mol Cell Biol* 17: 6609–6617.
40. Smith CL, Onate SA, Tsai MJ, O'Malley BW (1996) CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription. *Proc Natl Acad Sci USA* 93: 8884–8888.
41. Gregory CW, Wilson EM, Apparao KB, Lininger RA, Meyer WR, Kowalik A, Fritz MA, Lessey BA (2002) Steroid receptor coactivator expression throughout the menstrual cycle in normal and abnormal endometrium. *J Clin Endocrinol Metab* 87: 2960–2966.
42. Wieser F, Schneeberger C, Hudelist G, Singer C, Kurz C, Nagele F, Gruber C, Huber JC, Tschugguel W (2002) Endometrial nuclear receptor co-factors SRC-1 and N-CoR are increased in human endometrium during menstruation. *Mol Hum Reprod* 8: 644–650.
43. Shiozawa T, Shih HC, Miyamoto T, Feng YZ, Uchikawa J, Itoh K, Konishi I (2003) Cyclic changes in the expression of steroid receptor coactivators and corepressors in the normal human endometrium. *J Clin Endocrinol Metab* 88: 871–878.
44. Serra MJ, Ledford BE, Baggett B (1979) Synthesis and modification of the histones during the decidual cell reaction in the mouse uterus. *Biol Reprod* 20: 214–220.
45. Sakai N, Maruyama T, Sakurai R, Masuda H, Yamamoto Y, Shimizu A, Kishi I, Asada H, Yamagoe S, Yoshimura Y (2003) Involvement of histone acetylation in ovarian steroid-induced decidualization of human endometrial stromal cells. *J Biol Chem* 278: 16675–16682.
46. Uchida H, Maruyama T, Nagashima T, Asada H, Yoshimura Y (2005) Histone Deacetylase Inhibitors Induce Differentiation of Human Endometrial Adenocarcinoma Cells through Up-regulation of Glycodelin. *Endocrinology* 146: 5365–5373.
47. Uchida H, Maruyama T, Ono M, Ohta K, Kajitani T, Masuda H, Nagashima T, Arase T, Asada H, Yoshimura Y (2007) Histone deacetylase inhibitors stimulate cell migration in human endometrial adenocarcinoma cells through up-regulation of glycodelin. *Endocrinology* 148: 896–902.
48. Uchida H, Maruyama T, Ohta K, Ono M, Arase T, Kagami M, Oda H, Kajitani T, Asada H, Yoshimura Y (2007) Histone deacetylase inhibitor-induced glycodelin enhances the initial step of implantation. *Hum Reprod* 22: 2615–2622.
49. Bergamini CM, Pansini F, Bettocchi S, Jr., Segala V,

Dallocchio F, Bagni B, Mollica G (1985) Hormonal sensitivity of adenylate cyclase from human endometrium: modulation by estradiol. *J Steroid Biochem* 22: 299–303.

50. Tanaka N, Miyazaki K, Tashiro H, Mizutani H, Okamura H (1993) Changes in adenylyl cyclase activity in human endometrium during the menstrual cycle and in human decidua during pregnancy. *J Reprod Fertil* 98: 33–39.
51. Tang B, Gurpide E (1993) Direct effect of gonadotropins on decidualization of human endometrial stroma cells. *J Steroid Biochem Mol Biol* 47: 115–121.
52. Telgmann R, Maronde E, Tasken K, Gellersen B (1997) Activated protein kinase A is required for differentiation-dependent transcription of the decidual prolactin gene in human endometrial stromal cells. *Endocrinology* 138: 929–937.
53. Gellersen B, Kempf R, Telgmann R (1997) Human endometrial stromal cells express novel isoforms of the transcriptional modulator CREM and up-regulate ICER in the course of decidualization. *Mol Endocrinol* 11: 97–113.
54. Labied S, Kajihara T, Madureira PA, Fusi L, Jones MC, Higham JM, Varshochi R, Francis JM, Zoumpoulidou G, Essafi A, Fernandez de Mattos S, Lam EW, Brosens JJ (2006) Progestins regulate the expression and activity of the forkhead transcription factor FOXO1 in differentiating human endometrium. *Mol Endocrinol* 20: 35–44.
55. Pohnke Y, Schneider-Merck T, Fahnenstich J, Kempf R, Christian M, Milde-Langosch K, Brosens JJ, Gellersen B (2004) Wild-type p53 protein is up-regulated upon cyclic adenosine monophosphate-induced differentiation of human endometrial stromal cells. *J Clin Endocrinol Metab* 89: 5233–5244.
56. Levine AJ (1997) p53, the cellular gatekeeper for growth and division. *Cell* 88: 323–331.
57. Schneider-Merck T, Pohnke Y, Kempf R, Christian M, Brosens JJ, Gellersen B (2006) Physical interaction and mutual transrepression between CCAAT/enhancer-binding protein beta and the p53 tumor suppressor. *J Biol Chem* 281: 269–278.
58. Hu W, Feng Z, Teresky AK, Levine AJ (2007) p53 regulates maternal reproduction through LIF. *Nature* 450: 721–724.
59. Wagner BL, Norris JD, Knotts TA, Weigel NL, McDonnell DP (1998) The nuclear corepressors NCoR and SMRT are key regulators of both ligand- and 8-bromo-cyclic AMP-dependent transcriptional activity of the human progesterone receptor. *Mol Cell Biol* 18: 1369–1378.
60. Rowan BG, Garrison N, Weigel NL, O'Malley BW (2000) 8-Bromo-cyclic AMP induces phosphorylation of two sites in SRC-1 that facilitate ligand-independent activation of the chicken progesterone receptor and are critical for functional cooperation between SRC-1 and CREB binding protein. *Mol Cell Biol* 20: 8720–8730.
61. Uchikawa J, Shiozawa T, Shih HC, Miyamoto T, Feng YZ, Kashima H, Oka K, Konishi I (2003) Expression of steroid receptor coactivators and corepressors in human endometrial hyperplasia and carcinoma with relevance to steroid receptors and Ki-67 expression. *Cancer* 98: 2207–2213.
62. Abdel-Hafiz H, Takimoto GS, Tung L, Horwitz KB (2002) The inhibitory function in human progesterone receptor N termini binds SUMO-1 protein to regulate autoinhibition and transrepression. *J Biol Chem* 277: 33950–33956.
63. Johnson ES (2004) Protein modification by SUMO. *Annu Rev Biochem* 73: 355–382.
64. Jones MC, Fusi L, Higham JH, Abdel-Hafiz H, Horwitz KB, Lam EW, Brosens JJ (2006) Regulation of the SUMO pathway sensitizes differentiating human endometrial stromal cells to progesterone. *Proc Natl Acad Sci USA* 103: 16272–16277.
65. Hunter T (1998) The Croonian Lecture 1997. The phosphorylation of proteins on tyrosine: its role in cell growth and disease. *Philos Trans R Soc Lond B Biol Sci* 353: 583–605.
66. Maruyama T, Yoshimura Y, Yodoi J, Sabe H (1999) Activation of c-Src kinase is associated with *in vitro* decidualization of human endometrial stromal cells. *Endocrinology* 140: 2632–2636.
67. Yamamoto Y, Maruyama T, Sakai N, Sakurai R, Shimizu A, Hamatani T, Masuda H, Uchida H, Sabe H, Yoshimura Y (2002) Expression and subcellular distribution of the active form of c-Src tyrosine kinase in differentiating human endometrial stromal cells. *Mol Hum Reprod* 8: 1117–1124.
68. Shimizu A, Maruyama T, Tamaki K, Uchida H, Asada H, Yoshimura Y (2005) Impairment of decidualization in SRC-deficient mice. *Biol Reprod* 73: 1219–1227.
69. Thomas SM, Brugge JS (1997) Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol* 13: 513–609.
70. Maruyama T, Yamamoto Y, Sakai N, Shimizu A, Shimoki A, Masuda T, Yoshimura Y (2002) Protein tyrosine phosphorylation signaling in the differentiation of human endometrial stromal cells. *Keio J Med* 51: 93–99.
71. Maruyama T, Yamamoto Y, Sakai N, Sakurai R, Shimizu A, Hamatani T, Yoshimura Y (2002) Protein tyrosine kinases and tyrosine phosphorylation in differentiating human endometrial stromal cells. In: Maruo, T., Barlow, D., Mardon, H. and Kennedy, S. (eds) Cell and Molecular Biology of Endometrium in Health and Disease. Soeisha, Osaka: 94–106.
72. Maruyama T, Yamamoto Y, Shimizu A, Masuda H, Sakai N, Sakurai R, Asada H, Yoshimura Y (2004) Pyrazolo pyrimidine-type inhibitors of SRC family

tyrosine kinases promote ovarian steroid-induced differentiation of human endometrial stromal cells *in vitro*. *Biol Reprod* 70: 214–221.

73. Nagashima T, Maruyama T, Uchida H, Kajitani T, Arase T, Ono M, Oda H, Kagami M, Masuda H, Nishikawa S, Asada H, Yoshimura Y (2008) Activation of SRC kinase and phosphorylation of STAT5 are required for decidual transformation of human endometrial stromal cells. *Endocrinology* 149: 1227–1234.
74. Maruyama T, Yoshimura Y, Sabe H (1999) Tyrosine phosphorylation and subcellular localization of focal adhesion proteins during *in vitro* decidualization of human endometrial stromal cells. *Endocrinology* 140: 5982–5990.
75. Darnell JE, Jr. (1997) STATs and gene regulation. *Science* 277: 1630–1635.
76. Yoon MS, Koo JB, Hwang JH, Lee KS, Han JS (2005) Activation of phospholipase D by 8-Br-cAMP occurs through novel pathway involving Src, Ras, and ERK in human endometrial stromal cells. *FEBS Lett* 579: 5635–5642.
77. Shupnik MA (2004) Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. *Oncogene* 23: 7979–7989.
78. Migliaccio A, Piccolo D, Castoria G, Di Domenico M, Bilancio A, Lombardi M, Gong W, Beato M, Auricchio F (1998) Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. *EMBO J* 17: 2008–2018.
79. Scheid MP, Woodgett JR (2001) PKB/AKT: functional insights from genetic models. *Nat Rev Mol Cell Biol* 2: 760–768.
80. Nicholson KM, Anderson NG (2002) The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* 14: 381–395.
81. Guzeloglu Kayisli O, Kayisli UA, Luleci G, Arici A (2004) *In vivo* and *in vitro* regulation of Akt activation in human endometrial cells is estrogen dependent. *Biol Reprod* 71: 714–721.
82. Yoshino O, Osuga Y, Hirota Y, Koga K, Yano T, Tsutsumi O, Taketani Y (2003) Akt as a possible intracellular mediator for decidualization in human endometrial stromal cells. *Mol Hum Reprod* 9: 265–269.
83. Toyofuku A, Hara T, Taguchi T, Katsura Y, Ohama K, Kudo Y (2006) Cyclic and characteristic expression of phosphorylated Akt in human endometrium and decidual cells *in vivo* and *in vitro*. *Hum Reprod* 21: 1122–1128.
84. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96: 857–868.
85. Birkenkamp KU, Coffer PJ (2003) Regulation of cell survival and proliferation by the FOXO (Forkhead box, class O) subfamily of Forkhead transcription factors. *Biochem Soc Trans* 31: 292–297.
86. Mayo LD, Donner DB (2002) The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. *Trends Biochem Sci* 27: 462–467.
87. Chan RW, Schwab KE, Gargett CE (2004) Clonogenicity of human endometrial epithelial and stromal cells. *Biol Reprod* 70: 1738–1750.
88. Ludwig H, Spornitz UM (1991) Microarchitecture of the human endometrium by scanning electron microscopy: menstrual desquamation and remodeling. *Ann N Y Acad Sci* 622: 28–46.
89. Jabbour HN, Kelly RW, Fraser HM, Critchley HO (2006) Endocrine regulation of menstruation. *Endocr Rev* 27: 17–46.
90. Girling JE, Rogers PA (2005) Recent advances in endometrial angiogenesis research. *Angiogenesis* 8: 89–99.
91. Strauss III JF, Lessey BA (2004) The structure, function, and evaluation of the female reproductive tract. In: Strauss III, J.F. and Barbieri, R.L. ((ed) (eds)) Yen and Jaffe's Reproductive Endocrinology. Physiology, Pathophysiology, and Clinical Management. Elsevier Saunders 255–305.
92. Graubert MD, Ortega MA, Kessel B, Mortola JF, Iruela-Arispe ML (2001) Vascular repair after menstruation involves regulation of vascular endothelial growth factor-receptor phosphorylation by sFLT-1. *Am J Pathol* 158: 1399–1410.
93. Punyadeera C, Thijssen VL, Tchaikovski S, Kamps R, Delvoux B, Dunselman GA, de Goeij AF, Griffioen AW, Groothuis PG (2006) Expression and regulation of vascular endothelial growth factor ligands and receptors during menstruation and post-menstrual repair of human endometrium. *Mol Hum Reprod* 12: 367–375.
94. Gargett CE (2007) Uterine stem cells: what is the evidence? *Hum Reprod Update* 13: 87–101.
95. Padykula HA, Coles LG, Okulicz WC, Rapaport SI, McCracken JA, King NW, Jr., Longcope C, Kaiserman-Abramof IR (1989) The basalis of the primate endometrium: a bifunctional germinal compartment. *Biol Reprod* 40: 681–690.
96. Gargett CE, Chan RW, Schwab KE (2007) Endometrial stem cells. *Curr Opin Obstet Gynecol* 19: 377–383.
97. Schwab KE, Gargett CE (2007) Co-expression of two perivascular cell markers isolates mesenchymal stem-like cells from human endometrium. *Hum Reprod* 22: 2903–2911.
98. Ono M, Maruyama T, Masuda H, Kajitani T, Nagashima T, Arase T, Ito M, Ohta K, Uchida H, Asada H,

Yoshimura Y, Okano H, Matsuzaki Y (2007) Side population in human uterine myometrium displays phenotypic and functional characteristics of myometrial stem cells. *Proc Natl Acad Sci USA* 104: 18700–18705.

99. Masuda H, Maruyama T, Ono M, Nagashima T, Ito M, Okano H, Matsuzaki Y, Yoshimura Y (2005) Isolation and identification of putative stem/progenitor cells fro human cycling endometrium. The Endocrine Society's 87th Annual Meeting, OR52–54.
100. Grummer R (2006) Animal models in endometriosis research. *Hum Reprod Update* 12: 641–649.
101. Masuda H, Maruyama T, Hiratsu E, Yamane J, Iwanami A, Nagashima T, Ono M, Miyoshi H, Okano HJ, Ito M, Tamaoki N, Nomura T, Okano H, Matsuzaki Y, Yoshimura Y (2007) Noninvasive and real-time assessment of reconstructed functional human endometrium in NOD/SCID/γ_c^{null} immunodeficient mice. *Proc Natl Acad Sci USA* 104: 1925–1930.
102. Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K, Ueyama Y, Koyanagi Y, Sugamura K, Tsuji K, Heike T, Nakahata T (2002) NOD/SCID/γ_c^{null} mouse: an excellent recipient mouse model for engraftment of human cells. *Blood* 100: 3175–3182.
103. Contag CH, Bachmann MH (2002) Advances in *in vivo* bioluminescence imaging of gene expression. *Annu Rev Biomed Eng* 4: 235–260.
104. Masuda H, Okano H, Maruyama T, Yoshimura Y, Okano H, Matsuzaki Y (2008) *In vivo* imaging in humanized mice. *Curr Top Microbiol Immunol* 324: 179–197.

In Vivo Imaging in Humanized Mice

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Abstract The radiological modalities that are currently utilized as critical components in clinical medicine have also been adapted to small-animal imaging, among which are ultrasound imaging, X-ray computerized tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), and single-photon emission computed tomography (SPECT). Optical imaging techniques such as bioluminescence imaging (BLI) and fluorescence imaging (FLI) are approaches that are commonly used in small animals. Longitudinal surveys of living (i.e., nonsacrificed) animal models with these modalities provide some clues for the development of clinical applications. The techniques are absolutely essential

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for translational research. However, there are currently few tools available with sufficient spatial or temporal resolution ideal for all experimental studies. In this chapter, we provide a rationale and techniques for visualizing target cells in living small animals and an overview of the advantages and limitations of current imaging technology. Finally, we introduce a humanized mouse and a novel in vivo imaging system that we have developed. We also discuss real-time observations of reconstructs and clinical manifestations.

Abbreviations BLI: bioluminescence imaging; CBR luc: click beetle red-emitting luciferase; CCD: charge-coupled device; CT: x-ray computerized tomography; E_2: estradiol; E_2+ P_4: treatment with E_2 in combination with P_4; ^{18}FDG: 18-Fluorodeoxyglucose; FLI: fluorescence imaging; fMRI: functional MRI; FMT: fluorescence molecular tomography; γ-rays: gamma rays; GFP: green fluorescent protein; ICI: ICI-182,780; IRES : internal ribosomal entry site; MRI: magnetic resonance imaging; μMRI: micro-MRI; NIR: near-infrared range; NOG: NOD/ SCID/ γ_c^{null}; OVX: Ovariectomized; P_4: progesterone; PET: positron emission tomography; Q-Dot: quantum Dot; RF: radiofrequency; ROI: region of interest; SDECs: singly dispersed endometrial cells; SNR: signal-to-noise ratio; SPECT: single-photon emission computed tomography; $T_{1/2}$: half-time; UBM: ultrasound biomicroscopy; YFP: yellow fluorescent protein; 3D: three-dimensional

1 Introduction

Much of our understanding of various diseases has been obtained by using in vitro culture systems, in which the influences of intact organ structure, circulation, and the immune system have been removed. On the other hand, in vivo studies, especially those using humanized mice, can better mimic the actual physiological condition. In addition, in vivo imaging methods enable longitudinal studies of multiple processes and parameters in individual animals. Novel information about the specific three-dimensional (3D) locations, interaction, and dynamic states can be obtained through in vivo imaging without the necessity of killing the animals.

Accordingly, noninvasive and real time in vivo imaging in animal models including humanized mice holds promise in the provision of biomedical advances. In vivo imaging in humanized mice has become the significant bridge between in vitro basic research and clinical applications. The monitoring of cell growth and detection of dynamic states including metastasis in living animals have paved the way for the development of new drugs and expanded our knowledge of both the pathophysiology and the pathogenesis of each disease studied.

We have developed a mouse model for the study of the human endometrium and endometriosis. Our models are severe immunodeficient female mice transplanted with human endometrial cells. These diseased mice transplanted with human cells or tissues have proved very useful and have played a critical role in translational

research, and we think of these mice as one of the real "humanized mice". Furthermore, by application of bioluminescence imaging (BLI) techniques in our humanized mouse, the hormone-dependent behavior of the endometrium regenerated from lentivirally-engineered endometrial cells expressing a variant luciferase can be assessed noninvasively and quantitatively [32]. Our animal model will provide a powerful tool to study the physiology and pathophysiology of human endometrium and also to validate the effect of novel therapeutic agents and gene targeting on endometrium-derived diseases such as endometriosis.

2 Clinical Imaging Systems

2.1 Ultrasound Imaging

Ultrasound imaging, also called ultrasound scanning or sonography, is the most widely used cross-sectional imaging modality in the world [28], and not only medical doctors but also researchers are already trained in the basic principles of ultrasound imaging. Ultrasound images are captured in real-time, and they can show the size, structure, and movement of the internal organs in addition to blood flow. High-frequency sound waves are transmitted to the body, and the returning echoes are recorded to visualize the inside of the body.

Typical diagnostic ultrasound scanners operate in the frequency range of 2-15 MHz. The choice of frequency is a trade-off between spatial resolution and imaging depth. Lower frequencies produce less resolution but are able to image deeper into the body. Ultrasound examinations do not use ionizing radiation (such as X-rays) and avoid pain and tissue damage.

Moreover, ultrasonic visualization of living tissue at microscopic resolution is currently known as ultrasound biomicroscopy (UBM) [12], and UBM is in particular the key imaging tool for embryonic mouse research. UBM transducers operate at 40-100 MHz center frequencies, in contrast to diagnostic clinical transducers. Importantly, imaging issues that arise with high frequencies include loss of penetration, loss of depth of field, and changes in the ultrasound backscatter from blood [9, 12, 13, 42].

2.2 Computerized Tomography

Computerized tomography (CT) is an X-ray technique employing tomography in which digital geometry processing is used to image internal organs of the body. It is relatively safe, painless, and rapid. An X-ray tube, rotating around a specific area of the body, delivers an appropriate amount of X-radiation and takes pictures of that part of the internal anatomy from different angles. CT imaging relies on the

principle that various tissue types differentially absorb X-rays as they pass through the body. Modern scanners allow a large series of plane cross-sectional images to be reformatted in various planes or even as three-dimensional representations of structures. Electron beam CT (also called ultrafast CT) [36]is able to take pictures in a tenth of a second. It is useful in creating images of moving parts, such as the heart, without blurring.

CT systems for small animal have been developed specifically for high anatomic resolution imaging [3, 41]. As the relatively low X-ray photon-energy source of 25-50 keV is used, a high-resolution detector system rotates around the animal body to capture images. A typical scan of an entire mouse at a resolution of 100 μm would take about 15 min. Higher spatial resolution requires a longer period of scanning [25].

CT probes (probably iodine- or barium-based for X-ray contrast) have been designed for molecular imaging and used concurrently with CT scanning. Despite the superior soft tissue discrimination of animal scanner, poor soft tissue contrast still necessitates the use of a contrast agent to delineate clearly the internal organs of the animal. However, the use of contrast agent produces an ionization effect that results in radiation damage via superoxides and free radicals. The sensitivity and spatial resolution are dependent on the duration of radiation exposure (scanning time) and the amount of contrast agent used, which respectively affect the body being imaged [25].

2.3 Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is a diagnostic scanning system that measures the response of the atomic nuclei of body tissues to high-frequency radio waves when the tissues are placed in a strong magnetic field and that produces cross-sectional images of the internal organs. MRI uses a large magnet to generate a magnetic field around the subject. The magnetic field causes hydrogen atoms to align themselves in water and organic compounds, creating what is known as a magnetic dipole. The specific radiofrequency (RF) coils inside the bore of the magnet generate a temporary RF pulse, capable of changing the alignment of these dipoles. Once the pulse ceases, the dipoles return or "relax" to their normal baseline alignment. The relaxation behavior of the dipoles is described by both T1 and T2 relaxation. Both parameters are different for different tissue, resulting in contrast in MRI imaging. Depending on the timing of sequence the contrast can be predominantly T1- or T2 weighted [25].

MRI is primarily used in medical imaging to demonstrate pathological or other physiological alterations of living tissues. MRI can extract not only structural information but also physiological and molecular information. These are helpful in the diagnosis of abnormalities without the possibly harmful effects of X-rays or other forms of radiation. MRI scans are very valuable in detecting and delineating tumors and in providing images of the brain, the spinal cord, the heart, and other soft-tissue

organs. The disadvantage is that it requires a longer scanning time than other computer-assisted forms of scanning, which makes it more sensitive to motion and of less value in scanning the chest or abdomen. Although the images are similar to those of CT scans, MRI images provide better contrast between normal and diseased tissue than those produced by other computer-assisted imaging.

When it is used to provide a dynamic picture of oxygen metabolism during specific mental activities, it is called functional MRI (fMRI) [11]. This shows changes of local blood flow and hemoglobin oxygenation in response to altered neuronal activity. This change correlates with levels of neuronal activity in specific brain regions, and therefore fMRI allows mapping of functional centers of the brain.

High-resolution MRI is widely used in small animals (micro-MRI, μMRI), and reveals fine morphological details [37]. Stronger magnetic fields can be used and higher spatial resolution (25-50 μm) with exquisite morphological detail can be achieved, but for the most part requires field strengths of 7-11 T and long acquisition times (hours-typically overnight). Advantages of μMRI include its noninvasive nature (low toxicity), excellent tissue contrast, and ability to reconstruct images in any plane, including 3D reconstruction [42].

Recently, developments in animal MRI have focused on the development of new contrast agents that increase sensitivity and specificity. Contrast agents can be classified as nonspecific, targeted, and smart probes [4]. Nonspecific probes such as gadolinium chelates show a nonspecifically distributed pattern and are used to measure tissue perfusion and vascular permeability. Targeted probes such as gadolinium-labeled avidin and annexin V-supramagnetic iron oxide nanoparticles are designed to specifically bind to ligands such as peptides and antibodies. Smart probes tag a specific ligand similar to targeted agents but differ in that the probe signal changes on interaction with the specific ligand [25].

2.4 Single-Photon Emission Computed Tomography

Single-photon emission computed tomography (SPECT) is a nuclear medicine tomographic imaging technique using gamma rays (γ-rays). It allows us to visualize blood flow and metabolism. A radioactive isotope is attached to a substance that is easily taken up by target cells. As the isotope breaks down, it releases energy in the form of γ-rays. The γ-rays are like beacons of light that signal where the compound is in the body and are acquired by a gamma camera from multiple angles. A computer then translates these data to yield a 3D data set. This data set can be freely reformatted or manipulated to show cross-sectional slices along any chosen axis of the body.

In contrast to clinical use, small animal imaging require higher spatial resolution. This is achieved by pinhole collimators, and SPECT systems for small animals have appeared in recent years [2, 22].

SPECT is similar to a positron emission tomography (PET) scan at first glance, but it differs from PET scans in that isotopes are direct gamma emitters in a single

direction, necessitating different instrumentation for detection, which results in a limitation to the detection efficiency (to around 10^{-4} of number of γ-rays) of SPECT. Longer-lived radioactive isotopes, including ^{111}In, ^{123}I, ^{125}I, ^{201}TI, and ^{99m}Tc, are typically used [27, 45].

2.5 Positron Emission Tomography

PET is a clinical imaging technique that monitors metabolic, or biochemical, activity in the brain and other organs by tracking the movement and concentration of a radioactive tracer injected into the bloodstream. The radioactive atoms used in a PET scan emit subatomic particles called positrons (positive electrons), which collide with their negatively charged counterparts, namely, electrons. The two particles annihilate each other and emit two 511-keV photons (γ-rays) that radiate in opposite directions and can be recorded by a ring of detectors round the body and traced back to their point of origin. The acquired data are organized by a computer into 3D data sets to produce two-dimensional slices for all angular views.

In the past, PET was only used for large animals. However, because of technological innovation and improved imaging resolution in recent years, micro-PET scanners have been developed for small-animal imaging [16, 20]. The major limitations of PET are its spatial resolution and image noise. Spatial resolution of PET scans is typically about 2^3 mm^3 [8]. Newer-generation scanners can achieve a resolution of about 1^3 mm^3 [6].

There are many PET isotopes with different half-times ($T_{1/2}$) from minutes to days, for example, ^{15}O, ^{13}N, ^{11}C, ^{18}F, ^{64}Cu, and ^{124}I [27, 45]. A well-known example of an isotope, 18-fluorodeoxyglucose (^{18}FDG), is widely applied in tumor studies. It accumulates in tumor-specific sites because tumor cells have greater glucose uptake rate and glycolytic metabolism than normal tissues [17]. Many other radiopharmaceuticals can be engineered, most commonly from "biologic" positron emitters such as ^{18}F and ^{11}C, to target specific molecular targets within defined in vivo biochemical pathways and processes [7].

3 Optical Imaging Systems

Optical imaging systems have been developed that use both bioluminescent [44] and fluorescent [15] signals. This technique employs quantitative light emission, namely, photons, to obtain measurements of relevant biological parameters, including proteins and nucleic acids. Further advancement has come through the development of new targeted bioluminescent probes, near-infrared fluorochromes, and red fluorescent proteins [52]. To detect low levels of light or photons, a very sensitive charge-coupled device (CCD) detector is used [21]. The CCD detector is silicon-based and is capable of detecting light from the visible range (395- 600 nm) to the

near-infrared range (NIR: 600-2,500 nm) [25]. This imaging modality shows attractive data from organs close to skin in small animals, but this is likely to remain restricted to relatively superficial targets because of the absorbing and scattering properties of tissue in the visible range and NIR [26].

3.1 Fluorescence Imaging

Fluorescence is light of a visible color emitted from a substance under stimulation or excitation by light or other forms of electromagnetic radiation. The light is given off only while the stimulation continues. Visible light is used to excite fluorescence within the subject, and a camera or fluorescence microscopy system detects the emitted light from the region of interest. The commonly used strategy is to fluorescently tag the cells, tissue, or molecules under investigation with substances known to fluoresce. The most popular fluorochrome is green fluorescent protein (GFP), which is derived from the jellyfish *Aequorea victoria*. The wild-type GFP emits light at 509 nm, whereas its variant EGFP has a longer emitting wavelength and is 35-fold brighter [24]. Thus there are a lot of fluorescent proteins, and increasing numbers of new bright fluorescent probes with a variety range of emission wavelengths and greater stability have been developed and are now available [46].

Fluorochromes of wavelengths greater than 600 nm should be used in order to minimize absorbance by surrounding tissue and to distinguish background and autofluorescence [49]. Indeed, the use of NIR fluorochromes achieves maximum tissue penetration and minimum background and autofluorescence [53], and several applications have exploited the NIR range [29] with best results typically achieved when the emission wavelengths of the dye are between 500 and 950 nm [30].

3.2 Bioluminescence Imaging

Bioluminescence is biochemical emission of light, with very little heat, by living organisms such as fireflies and deep-sea fishes as the result of a chemical reaction during which chemical energy is converted to light energy. This reaction is mediated by one of the luciferase family of photoproteins that can be isolated either from the sea pansy (*Renilla reniformis*) or from the North American firefly (*Photimus pyralis*). Different organisms produce different bioluminescent substances and use different substrates. Marine bioluminescent organisms use coelenterazine as a substrate, and terrestrial organisms use d-luciferin, which provides a longer-lived and longer wavelength. Luciferase is normally bound to ATP (adenosine triphosphate) in an inactive form, but in catalysis of luciferin it is liberated from the ATP and combines with oxygen to form an oxyluciferin in an excited state, which quickly decays, emitting photons of visible light as it does [54].

As in the case of fluorescence imaging (FLI), the use of luciferases that have a significant portion of their emission greater than 600 nm, such as luciferase derived from fireflies and click beetles (approximately 60% of the light emitted from these two enzymes has wavelengths greater than 600 nm), will lead to more sensitive detection of the labeled cells in vivo [38, 55].

In vivo bioluminescence imaging (BLI) has been applied in the assessment of the extent of tumor growth and response to therapy by transplantation of tumor cells transfected to express luciferase into animals [47]. Furthermore, the expression of luciferase can be controlled so that it is only expressed when a gene of interest is being transcribed [5, 31]. Cells expressing luciferase in animal models can be easily identified through their emission of light in the range of 400-620 nm by administration of luciferin [19]. This technology has become an invaluable tool that has been employed to dynamically monitor tumor growth or transcriptional activity in living animals.

3.3 Comparison of BLI *with* FLI

First of all, as the luciferase reaction is energy-dependent and requires ATP and oxygen, the luminescent signal is produced only from living cells, which are different from the fluorescent signals that can be sustained even in nonliving cells. The major attraction of BLI over FLI is that, although absolute light levels generated by the targets may be low, photons are generated generally only where luciferase is present, leading to an extremely low level of background signals and excellent signal-to-noise ratios (SNRs). In contrast, FLI requires an external light source to stimulate the emission of light from the probe, and the light source could also generate bright background signals arising from the animal's intrinsic autofluorescence [26, 44, 50].

However, there are some limitations in BLI. First, light transmission efficiency is dependent on the type and location of tissue being assessed because of the narrow range, from 400 nm to 620 nm, of the light emission peak. Highly vascular organ structures contain hemoglobin that absorbs transmitted light, which results in about a ten-fold reduction of the bioluminescent signal for every centimeter of tissue depth [10]. Second, because the catalytic reaction in BLI is time- and enzyme-dependent, the window period for optimum image capture must be determined [25].

In contrast, a distinctive advantage of FLI over BLI is that it does not require administration of a substrate for visualization. FLI may be more convenient and allow easier capture of images at multiple time points, since administration of a substrate into the animal is not required. In addition, FLI is a more flexible technology, since it permits the use of a far wider range of probes, labeling methods, and targets. The number of photons emitted in FLI is orders of magnitude greater than that in BLI [26, 50].

Among the great number of fluorescent probes, Quantum Dots (Q-Dots), which are semiconductor nanocrystals that have long-term stability and fluoresce brightly up to the NIR spectrum on excitation, have been developed for imaging [1]. Q-dots enable antibody-targeted spectral imaging and analysis such as examining the

distribution of Q-dot-labeled antitumor antibodies in mice [14]. In addition, this method will be available as multimodal contrast agents for not only FLI but also PET or MRI detection [33]. An agent such as Q-Dots is valuable in multimodality imaging as described below.

4 Multimodality Imaging

Imaging modalities can be divided into two groups. One group includes ultrasound, CT, and MRI, providing structural information, while another group includes SPECT, PET, and optical imaging, providing functional or molecular information. Both groups have some drawbacks and advantages (Tables 1 and 2). Therefore, the combination of different imaging modalities has been developed to offset the disadvantage of each modality, and it will develop into a powerful tool with the recent development of contrast agents including Q-Dots as mentioned in the previous section.

PET/CT has only recently developed to enable accurate diagnosis. By combining the structural anatomic information of CT scans with the metabolic or cellular activity data of PET scans, it has become possible to visualize anatomy and function simultaneously. The anatomic information enables compensation of the correlated radionuclide data for physical perturbations such as photon attenuation, scatter radiation, and partial volume errors. Thus, dual-modality imaging provides a priori information that can improve both the visual quality and the quantitative accuracy of the radionuclide images. The hybrid imaging of PET/CT has been shown to improve not only the sensitivity of PET interpretation but also its specificity. Micro-PET/CT is used for small animals to obtain high anatomic resolution with functional information [48, 49]. The combination of the various modalities, called multimodality imaging, offers valuable information. By the same token, SPECT/CT [18] and optical PET [43] have been developed in addition to PET/CT.

A further development in FLI is fluorescence molecular tomography (FMT), which has been developed for acquiring images of fluorescently labeled proteins and for deeper targets. It employs continuous wave or pulsed light from different sources to excite the fluorochrome label, and multiple detectors are arranged spatially around the subject analogous to the set-up in CT or MR scanners [39, 40]. Computation of the data generates a 3D image. The resulting images have a resolution of 1-2 mm, and the fluorochrome detection threshold is in the nanomolar range [25].

5 A Novel In Vivo Imaging System of the Endometrial Model Mouse

As the bioluminescent signal is emitted only from living cells expressing luciferase, leading to low background noise, we chose BLI as the modality to visualize an artificial menstrual cycle in mice and developed an in vivo imaging system based on this concept.

Table 1 The properties of currently available in vivo imaging techniques

Modality	Basis	Reagents	Acquisition time	Tissue penetration depth	Spatial resolution	Signal quantification capabilities	Cost (equipment and usage)
Ultrasound	High-frequency sound waves	Microbubbles	minutes	1–200 mm	50–500 μm (animal) 0.1–1 mm (clinical)	Low	Low
CT	X-rays	Iodine	minutes	No limit	30–50 μm (animal) 0.5–1 mm (clinical)	N/A	Medium-high
MRI	Radio frequency waves	Paramagnetic cation probes	minutes–hours	No limit	25–100 μm (animal) 0.2 mm (clinical)	Medium	High
PET	High energy γ-rays	^{18}F, ^{11}C, ^{13}N, ^{15}O, ^{124}I, ^{94m}Tc labeled probes or substrates for reporter transgenes	minutes	No limit	1–2 mm (animal) 6–10 mm (clinical)	High	High
SPECT	Low energy γ-rays	^{99m}Tc, ^{111}In, ^{125}I labeled probes	minutes	No limit	1–2 mm (animal) 7–15 mm (clinical)	Medium-high	Medium-high
FLI	Visible to near-infrared light	Fluorescent proteins, fluorescent dyes, and quantum dots (semiconductor)	seconds–minutes	1–20 mm	1–10 mm (animal) (depending on tissue depth)	Low-medium	Low
BLI	Visible light	Luciferase and substrates (luciferin, coelenterazine)	seconds–minutes	1–10 mm	1–10 mm (animal) (depending on tissue depth)	Low-medium	Low

Table 2 Advantages and disadvantages of in vivo imaging modalities

Modality	Advantages	Disadvantages
Ultrasound	Real-time imaging, low cost, and user-friendly	Limited ability to image through bone or lungs
CT	Good anatomic resolution	Relatively poor soft-tissue contrast Radiation to animal with CT contrast agents
MRI	Highest spatial resolution The ability to combine functional information and anatomic details	Low sensitivity Long acquisition time Long image processing time
PET	High sensitivity The ability of quantitative measure Variety of probes and strategies confers a high degree of versatility	Low resolution Unincorporated substrate can increase noise Cyclotron required to generate short-lived radioisotope Radiation to animal
SPECT	Multiple probes can be detected simultaneously Radioisotopes have longer half-lives than those used in PET	10–100x less sensitive than PET Relative low resolution Radiation to animal
FLI	High sensitivity Easy and quick to image Multiple reporter wavelengths enables multiplex imaging Detect fluorochromes in live and dead cells Transgene-based approach confers versatility	Prone to attenuation with increased tissue depth Probes with emission wavelength <600 nm prone to autofluorescence of nonlabeled cells
BLI	High sensitivity Easy and quick to image Provides relative measure of cell viability or function Available for gene expression and cell tracking Transgene-based approach confers versatility	Low anatomic resolution Light emission prone to attenuation with increased tissue depth

5.1 Lentiviral Introduction of Reporter Genes into Primary Endometrial Cells

We have developed a recombinant lentivirus capable of introducing and stably expressing both the Venus [a yellow fluorescent protein (YFP) mutant] [35] gene and the click beetle red-emitting luciferase (CBR luc, a luciferase variant) [55] gene in the targeted cells (Fig. 1a). These two reporter markers are useful for

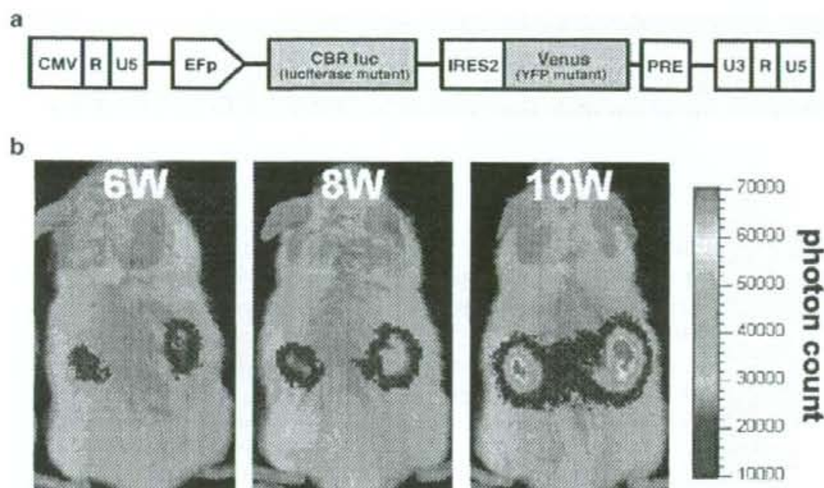

Fig. 1 Lentiviral construct for fluorescence and bioluminescence and optical bioluminescence images of the growth of the tissues reconstructed from lentivirally transduced SDECs in living NOG mice. **a** Lentiviral construct encoding a dual function CBR luc (a luciferase variant) and Venus (a YFP variant) bicistronic reporter gene connected via an internal ribosomal entry site (*IRES*). **b** Representative sequential BLI of NOG mice treated for different durations with two E_2 pellets

flow cytometry sorting and the detection of living cells from outside the body, respectively. Indeed, fluorescence microscopy-revealed Venus signals were detected in lentivirally-infected cells, and these cells could be sorted easily by flow cytometry. Moreover, CBR luc (maximum emission at 613 nm) has the potential to pass through thicker tissue. By combining the advantages of the lentivirus and CBR luc, we successfully assessed the dynamic state of the endometrial reconstructs in living NOD/SCID/γ_c^{null} (NOG) mice [23].

5.2 *Noninvasive, Real-Time, and Quantitative Assessment of the Reconstructs by BLI*

We transplanted singly dispersed endometrial cells (SDECs) beneath the kidney capsules of severely immunodeficient mice, NOG mice, and we demonstrated for the first time that a functional endometrium-like structure can be regenerated from SDECs [32]. This model is the humanized mouse we developed. To apply this humanized mouse to an in vivo BLI system, we transplanted the human endometrial cells infected with our above-mentioned lentivirus beneath the kidney capsule. Consequently, sequential BLI of the ventrally positioned estradiol (E_2)-treated NOG mouse 6-10 weeks after xenotransplantation revealed bioluminescent (CBR) signals in locations corresponding to the bilateral kidneys (Fig. 1b).