of transcription 5 (STAT5), CCAAT enhancer-binding proteins (C/EBPs), and forkhead box O (FOXO1) [16–18]. By hijacking these transcription factors, the activated PR acquires control of the diverse gene families involved in decidualization.

ii. Non-genomic action

Progesterone, like other steroid hormones, can trigger rapid cytoplasmic events that are independent of its genomic actions [19]. The molecular basis of this was unknown until the recent discovery of a family of membrane progestin receptors (mPR-α, mPR-β, mPR-γ) [20, 21]. Though expression of these mPRs has been identified in the human endometrium [22], their precise role in hESC differentiation is unclear. Alternatively, it has been suggested that the rapid, non-genomic effects of progesterone are mediated by binding of a cytoplasmic PR to the SH3 domain of SRC, a non-receptor type tyrosine kinase. Binding results in phosphorylation and activation of the p42/44 MAPK/ERK signal transduction pathway downstream of SRC [23]. The ability of the PR to activate kinase cascades shows that the PR is not only capable of acting as a transcription factor, but also may directly activate signaling pathways from the cytoplasm. The role of SRC signaling in decidualization will be addressed later in this article.

PR-associated decidual proteins

There is growing evidence suggesting a role for the PR as a platform for the formation of a decidua-specific transcriptional complex involving such diverse transcription factors as FOXO, C/EBPβ, STAT5, and co-activators [8].

i. PR and C/EBPβ

Since PRL is one of the major products of decidualized hESCs, the decidual PRL (dPRL) promoter has been exploited as a tool to identify transcription factors relevant to decidualization. Analysis of this promoter reveals that the dPRL-332/-270 promoter element contains a PR binding half-site adjacent to the C/EBP binding sites [8]. Among the C/EBPs, C/EBPβ is the predominant form in decidualized stromal cells [24]. C/EBPβ is essential for female reproduction; its absence hampers ovulation, breast development and function [25–27]. Taking advantage of this information, Christian *et al.* demonstrated that the PR can physically associate with the two C/EBPβ isoforms [17]: the full-length liver-enriched activating protein (LAP) and the truncated liver-enriched inhibitory protein (LIP). LIP lacks the N-terminal transactivation domains of LAP and acts as a potent repressor of C/EBP-dependent transcription [28].

The functional consequences of this interaction are dependent upon the relative ratios of PR and C/EBPβ isoforms in the cell [17]. Transfection studies demonstrate that PR-A, but not PR-B, greatly enhances LAP-dependent activation of the dPRL –332/–270 promoter region in a ligand-dependent manner. Conversely, overexpression of LIP, but not LAP, enhances PR-B transactivation of single and complex progesterone response element-dependent promoters [17]. Western blot analysis studies show that only LAP is present in normal non-pregnant human endometrium [29]. Intriguingly, C/EBPβ is also involved in the transcriptional regulation of the IGFBP-1 promoter [30], another representative decidual marker.

ii. PR and FOXO1

The FOXO proteins constitute a subclass of the winged helix/Forkhead box class of transcription factors. FOXO transcription factors are critical mediators in cell fate decisions in response to growth factor, hormonal and environmental cues [31]. Of the three human FOXO proteins (FOXO1, FOXO3a, and FOXO4), FOXO1 is markedly induced upon decidualization both *in vivo* and *in vitro*, and is involved in regulating the expression of decidual marker genes, such as PRL and IGFBP-1 [8, 18, 29, 32]. Indeed, FOXO1 enhances the activity of the dPRL promoter cooperatively with C/EBPβ through the discrete –332/–270 region, which also harbors the imperfect PR binding site [29]. FOXO1 also stimulates the IGFBP-1 promoter through direct interaction with HOXA10, a homeobox transcription factor [32].

iii. PR and STAT5

Members of the STAT family are activated by phosphorylation within the cytoplasm by diverse cell signaling pathways, including receptor-associated Janus kinases (JAKs) [33]. Phosphorylation of a conserved tyrosine residue in all STAT family members induces their dimerization and translocation to the nucleus. Within the nucleus, they regulate genes involved in the growth and differentiation of many tissues including adipocytes, hepatocytes, and mammary epithelial cells [33]. In the human endometrium, STAT5 is selectively expressed in the glandular epithelium. It is also expressed in a subset of stromal cells that also express the PRL receptor during the secretory phase, suggesting a potential role for STAT5 in differentiation [34].

STAT5 enhances the activity of the –332/–270 dPRL promoter region in hESCs [35]. Like C/EBP and FOXO1, STAT5 has also been shown to interact with the PR [16], which might contribute to the STAT5-mediated activation of the dPRL promoter region.

iv. Coactivators

Coactivators promote transcription initiation through protein interactions with components of the general transcription machinery and by promoting local remodeling of chromatin at specific promoters. The transcriptional coactivator CBP (CREB-binding protein) or its paralogue p300, was identified based on its ability to bind to CREB (cAMP-response element binding protein) [36]. It is now recognized as an integrator for a large number of transcriptional signals. It simultaneously interacts with diverse transcription factors and RNA polymerase II complexes, thus establishing contact between specific inputs and the basal transcription machinery [37, 38]. CBP/p300 interacts with C/EBPβ and enhances its activity [39]. C/EBPβ is also an important mediator of cAMP signaling in hESC, as will be outlined below. [24]. CBP is recruited to pre-initiation complexes containing steroid hormone receptors through the 160 kDa steroid receptor coactivator proteins (SRC-1/p160) [40]. The expression profiles of these coactivators and corepressors have been demonstrated in the endometrium throughout the menstrual cycle [41–43].

Nuclear receptor-associated coactivators including CBP/p300 and SRC-1 possess histone acetyltransferase activity (HAT), and histone acetylation has been implicated in decidualization [44]. Histone deacetylase inhibitors (HDACIs), which induce hyperacetylation of chromatin, facilitate the transcription of several genes. In this manner, they stimulate decidualization of human endometrial stromal cells *in vitro* [45]. In addition, HDACIs, through up-regulation of glycodelin, an implantation-related protein, also stimulate differentiation and cell motility of the endometrial epithelial cell line, Ishikawa [46, 47]. Thus, the increased levels of histone acetylation may contribute to implantation, one of the most essential functions of the endometrium. In support of this, we have recently demonstrated that glycodelin induction, following treatment with ovarian steroid hormones or an HDAC inhibitor, enhances implantation, as determined by an *in vitro* implantation assay using Ishikawa cells and the choriocarcinoma cell line, JAR [48].

Upstream signaling pathways regulate and cross-talk with PR signaling

Recently, it has become apparent that cAMP signaling regulates, modifies, and engages in cross-talk with the progesterone signaling pathway. cAMP signaling sensitizes human endometrial stromal cells to progesterone and eventually controls the expression and activity of a large number of transcription factors involved in decidualization [8].

cAMP signal transduction

cAMP is a ubiquitous second messenger molecule that is generated from adenosine triphosphate by adenylate cyclase. This enzyme is activated upon ligands binding to members of the family of G protein-coupled receptors (GPCRs) that are coupled with a stimulatory heterodimeric guanine nucleotide-binding protein (G protein). cAMP signaling is controlled at many levels. These include the receptor level, catabolism of cAMP by phosphodiesterases, modified composition of the PKA holoenzyme, expression of CREB and CREM (cAMP-response element modulator) isoforms with altered transcriptional activity, or a change in the expression level of coactivators or corepressors.

After ovulation, the endometrium is increasingly exposed to a variety of local and endocrine factors including prostaglandin E2, relaxin (RLX), CRH, LH, and FSH that are capable of stimulating cAMP production in hESCs. Activation of the cAMP pathway is the well established, initial and obligatory event that starts the decidual process *in vitro* [8]. In agreement, adenylate cyclase activity in the human endometrium increases during the menstrual cycle, and the cAMP content in biopsies obtained from patients during the secretory phase is higher than that in the proliferative phase [49, 50]. In pregnancy, the decidua is further exposed to a high level of hCG, which signals predominantly through the cAMP pathway [51].

In hESCs, RLX has the potential to modify the composition of the PKA holoenzyme, presumably resulting in a net increase in free, activated C protein and an increase in target protein phosphorylation [52]. Additionally, CREM isoforms and ICER (inducible cAMP early repressor) are involved in decidualization [53].

Downstream events of cAMP signaling

cAMP induces the expression or activation of several transcription factors, including FOXO1, STAT5,

and C/EBPβ, all of which are capable of interacting directly with the PR [16, 18, 29, 35, 54]. In addition, p53 is also up-regulated upon cAMP-induced differentiation of human endometrial stromal cells [55]. The promoters of dPRL and IGFBP-1 genes are activated by multimeric transcription factor complexes which assemble in response to an interplay of cAMP- and progesterone-dependent signals [8].

i. C/EBPβ

The C/EBP binding sites in the dPRL promoter are crucial for cAMP-induced activation, implicating C/EBPβ protein as a central mediator of the cAMP signal towards decidualization [24]. In agreement, C/EBPβ is not only induced by cAMP in cultured hESCs but also up-regulated *in vivo* in late secretory phase stromal cell nuclei [29].

ii. FOXO1

The expression and activity of FOXO1 itself is subject to intricate control mechanisms involving both the PKA pathway and the ligand-activated nuclear PR. Within three days of cAMP treatment, cultured hESCs up-regulate FOXO1 mRNA and protein. This response is markedly enhanced by progestin, although treatment with progestin alone does not induce FOXO1 expression [54]. In a manner strikingly parallel to C/EBPβ, FOXO protein accumulates in the nuclei of decidualized stromal cells *in vivo* [8]. Eventually, FOXO1 and C/EBPβ physically interact and cooperatively activate the dPRL promoter [29].

iii. STAT5

STAT5 is also a cAMP-induced transcription factor in decidualizing ESCs that has also been shown to interact with PR [35]. Treatment of primary ESC cultures with cAMP, with or without progestin, for two or more days results in induction, phosphorylation, dimerization, and nuclear translocation of STAT5 [35]. Induction of the dPRL promoter by cAMP plus progestin is markedly enhanced by STAT5 through its nuclear translocation and interaction with PR. This is abolished by coexpression of a dominant negative mutant of STAT5 [35].

iv. p53

The tumor suppressor protein p53 is a transcription factor that is present at extremely low levels in normal cells. In response to genotoxic stress, p53 protein is stabilized and rapidly accumulates. This ultimately leads to cell cycle arrest and DNA repair or to induction of apoptosis in damaged cells [56]. Thus, p53 exerts its biological function as the cellular gatekeeper for growth and division by transactivating cell cycle genes [56]. A massive and sustained up-regulation of p53 occurs during cAMP-induced decidualization of cultured hESCs [55]. Furthermore, a direct physical association with transrepression occurs between p53 and C/EBPβ [57]. Although the precise role of p53 in human decidualization remains elusive, in mice, p53 has recently been shown to be essential for regulating maternal reproduction, in particular, implantation, through leukemia-inhibitory factor (LIF) production [58].

v. Coactivators and Corepressors

cAMP activation of the PKA pathway disrupts the interaction of the PR with the corepressors NCoR and SMRT [59], thereby facilitating recruitment of the coactivator SRC-1 [60]. These corepressors and SRC-1 are all present in the human endometrium [61]; however, how cAMP regulates their behavior remains to be elucidated.

vi. PR and the SUMO pathway

Like many other transcription factors and cofactors, PR-A and PR-B are rapidly modified by small ubiquitin-like modifier (SUMO) -1 upon ligand binding [62].

Sumoylation denotes a process whereby SUMO covalently binds to target proteins, mostly transcription factors. Sumoylation profoundly changes the subnuclear localization, interactions, and activities of transcription factors [63]. Sumoylation often confers repressive properties. Intriguingly, cAMP signaling in ESCs alters the expression of many SUMO enzymes, resulting in a gradual loss of PR-A sumoylation and increased PR activity [64].

Other signaling pathways

i. SRC signal transduction

a. SRC activation and its essential role in decidualization

Many surface receptors for growth factors and cytokines possess tyrosine kinase activity and/or associate with non-receptor type tyrosine kinases [65]. As decidualized endometrial stromal cells produce a myriad of cytokines and growth factors [1–3, 6], it is likely that tyrosine phosphorylation signaling may be deeply involved in decidualization.

To address this, we performed an immunoblot analysis using a phosphotyrosine antibody and found that there were several differences in the profiles of the phosphotyrosinyl proteins between non-decidualized

and decidualized stromal cells [66]. Furthermore, we found that tyrosine kinase activity of SRC was increased during *in vitro* decidualization [66]. Subsequent immunohistochemical studies of the human endometrium and pregnancy decidua revealed that the kinase-active form of SRC was strongly expressed in decidual cells in humans and mice [67, 68].

SRC is a non-receptor tyrosine kinase that associates with integrins and many surface receptors including those for growth factors, cytokines, and G-protein coupled receptors (GPCR) [69]. SRC becomes activated upon ligand binding, and converts the extracellular stimuli to intracellular signals [69]. SRC is tethered to perinuclear membranes, endosomes, and secretory vesicles, and the cytoplasmic face of the plasma membrane by an N-terminal myristoyl group [69]. The kinase activity of SRC is up-regulated by dephosphorylation of its negative regulatory tyrosine residue, tyrosine 527 (Y527, corresponding to Y530 in humans), located at the carboxyl terminus. Its kinase activity is enhanced by autophosphorylation of tyrosine 416 [69]. C-terminal SRC kinase (CSK) phosphorylates Y527 thereby inactivating SRC; whereas a number of phosphatases (PTPs) dephosphorylate Y527 thereby activating SRC [69]. hESCs produce several bioactive substances including PDGF, EGF, CSF-1, IGF, IL-11, angiotensin II, bradykinin, PAF, prolactin, and oncostatin M during decidualization [1–3, 6]. Interestingly, all of these soluble factors have the potential to function as ligands for the transmembrane receptors that can couple with and activate SRC [70, 71]; therefore, it seems reasonable that SRC activation is accompanied by decidualization.

To address the essential role of SRC in decidualization of hESCs, knockdown experiments were subsequently performed using specific inhibitors of the SRC family of kinases: 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1) and 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) [72]. These inhibitors, however, unexpectedly promoted decidualization together with paradoxical SRC activation [72] by a mechanism that remains to be elucidated. We therefore examined the role of SRC in murine decidualization. Src null mice showed no apparent decidual response in their uteri [68]. This result clearly demonstrates that Src activity is indispensable for an appropriate progesterone induced decidualization response in mice [68]; however, it remains unclear whether SRC and its kinase activity are essential for decidualization in humans. To clarify this point, we recently conducted experiments in which an adenovirus was used to introduce the dominant negative mutant of SRC into hESCs [73]. The elimination of SRC kinase activity by overexpression of the mutant almost completely inhibited *in vitro* decidualization. This indicates that SRC kinase activation is also essential for decidualization in humans [73]. These results together corroborate the phenotype in the mouse and establish the importance of hormone-mediated SRC kinase activation in decidualization across species.

b. Downstream events of SRC signaling in hESCs

Though signaling pathways downstream of SRC are well elucidated in various types of cells, they are not clearly described in hESCs. We previously reported that despite the activation of decidual SRC, focal adhesion kinase (FAK) and paxillin, both well established substrates of SRC and components of the focal adhesion complex [69], remain hypophosphorylated in decidualized hESCs [74]. These results indicate that FAK and paxillin may not be substrates of SRC in decidualizing hESCs. Treatment of primary hESC cultures with cAMP (with or without progestin) leads to induction, phosphorylation, dimerization, and nuclear translocation of STAT5, eventually enhancing the activity of the –332/–270 decidual PRL promoter region [8, 35]. Members of the STAT family including STAT5 are activated by phosphorylation within the cytoplasm by diverse cell signaling pathways, including receptor-associated Janus kinases (JAK) [33, 75]. However, the nuclear accumulation of phosphorylated STAT5 in hESCs is independent of JAK activity, suggesting that other activating kinase(s) may regulate decidual STAT5 [8, 35]. Very recently, we have demonstrated that STAT5 was phosphorylated on tyrosine 694, a well-known SRC phosphorylation site during decidualization [73]. Knockdown of SRC signaling by the SRC dominant negative mutant markedly attenuates phosphorylation of STAT5 [73]. These results collectively indicate that the SRC-STAT5 pathway is essential for the decidualization of hESCs.

c. Possible regulatory mechanism of SRC activation in decidualized ESCs

The upstream regulatory mechanisms of SRC activity have been well elucidated in a variety of cells [69]. SRC couples with cell surface receptors for many bioactive substances including cytokines and growth

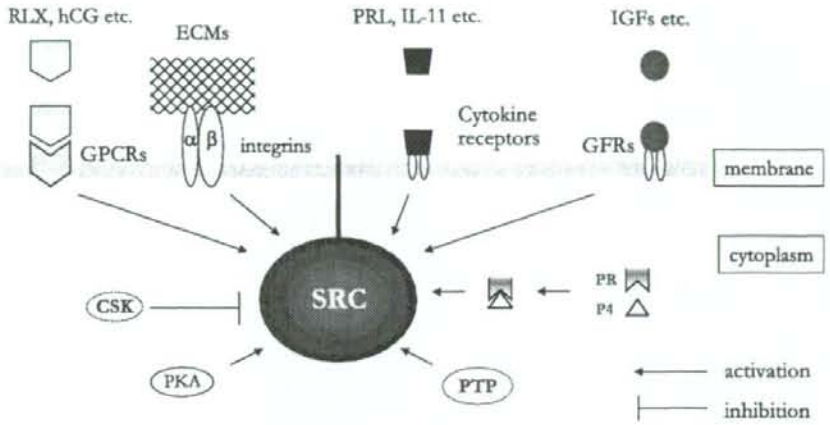

Fig. 1. Possible regulators of SRC activity in endometrial and decidual cells.
RLX, relaxin; hCG, human chorionic gonadotropin; GPCR, G protein-coupled receptors; ECMs, extracellular matrices; PRL, prolactin; IL-11, interleukin 11; IGFs, insulin-like growth factors; GFRs, growth factor receptors; CSK, c-terminal SRC kinase; PKA, protein kinase A; PR, progesterone receptor; PTP, protein tyrosine phoshphatase.

factors [69]; and these locally produced factors may activate SRC in an autocrine/paracrine manner. Indeed, IGF-I activates SRC in mouse endometrial cells [68]. In addition, SRC is activated by the cAMP/PKA signaling pathway in hESCs [76].

Progestins also positively regulate SRC activity [77]. They stimulate the SRC/MAPK pathway through indirect or direct interaction of ligand-bound progesterone receptors with SRC [23, 78]. This interaction may be facilitated when SRC becomes conformationally open upon dephosphorylation of tyrosine 527 (530 in human). In agreement, with this, we previously reported that decidual SRC becomes activated together with its dephosphorylation on tyrosine 530 [67, 72]. Furthermore, it is likely that SRC activation is hormone dependent in decidual hESCs, as withdrawal of E2 and progesterone reduces SRC kinase activity to its basal level and also changes the pattern of tyrosine phosphorylation to that of the unstimulated state [66]. Possible regulators of decidual SRC are illustrated in Fig. 1.

ii. PKB/AKT signal transduction

a. PKB/AKT signaling

The serine/threonine kinase (AKT), also known as protein kinase B (PKB), is the cellular homologue of the viral oncogene, v-Akt. It is phosphorylated and activated by multiple growth factors and functions as a downstream regulator of phosphoinositide 3-kinase (PI3K) signaling. Phosphorylated PKB/AKT is an important regulator of apoptosis and other multiple biological processes, including cell survival, the cell cycle, and glucose uptake [79, 80].

E_2 can directly and rapidly affect the PI3K-related signaling pathway by increasing the phosphorylation of PKB/AKT in endometrial cells [81]. This suggests that E_2 may exert part of its proliferative and anti-apoptotic effects by a non-genomic manner through the PKB/AKT signaling pathway. Progesterone/progestins counteract E_2 action at various molecular levels. In agreement, PKB/AKT becomes hypophosphorylated during progesterone-induced decidualization of hESCs *in vitro* [82, 83]. In contrast, phosphorylated AKT is strongly expressed in predecidual and decidual cells *in vivo* [83]. The discrepancy of the *in vivo* and *in vitro* results may be partly due to the production of decidualization-associated growth factors such as IGF-I that may activate PKB/AKT via PI3K.

b. Downstream events of PKB/AKT signaling

The transcriptional activity of FOXO proteins is critically regulated by their subcellular localization. Growth factor signaling through the PI3K pathway leads to phosphorylation of PKB/AKT that in turn phosphorylates downstream target proteins, including the FOXO transcription factors. Akt-dependent phosphorylation of nuclear FOXO results in its nuclear exclusion and inactivation [84, 85]. The observation that FOXO1 accumulates in the nuclei of cAMP-

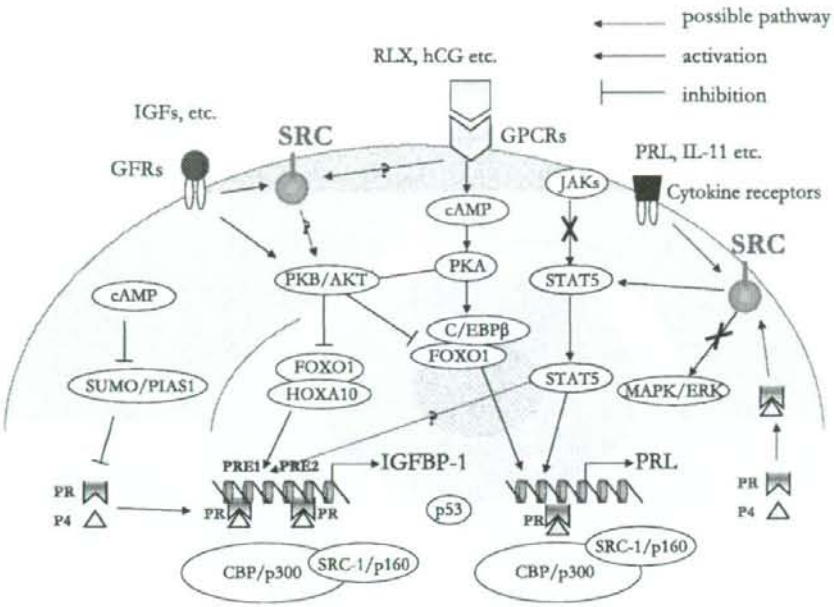

Fig. 2. Signaling events associated with decidualization.

treated ESC [8] suggests that the PI3K/PKB signaling pathway is suppressed upon decidualization. This is in agreement with previous reports that hypophosphorylation of PKB/AKT is tightly associated with *in vitro* decidualization of ESCs [82].

The PKB/AKT pathway also regulates the expression and localization of p53 [86]. Nuclear accumulation of p53 is the result of stabilization of p53 protein, rather than increased mRNA expression [86]. Proteasomal degradation of p53 is mediated by nuclear Mdm2 [86]. Nuclear translocation of Mdm2, in turn, is dependent on phosphorylation by PKB/AKT [86]. As expected, in cAMP-treated decidualized cells, p53 accumulation is associated with decreased nuclear Mdm2 and cytoplasmic PKB/Akt levels [55].

Thus, in addition to cAMP- and progestin-mediated signal transduction, other signaling pathways involving SRC and PKB/AKT, which are located downstream of the surface receptors for growth factors and cytokines, may serve to amplify and propagate the decidualization process in an autocrine or paracrine fashion. Signaling pathways responsible for decidualization are depicted in Fig. 2.

Regeneration of the human endometrium

After tissue breakdown and shedding of the differentiated endometrium (menstruation), the endometrium is programmed to regrow under the influence of E_2. The restructuring of the functional layer is critical to the development of a tissue ready for implantation or for menstruation. Vessel growth is particularly important in the endometrium of menstruating species where the spiral arterioles are a characteristic feature. These regeneration processes are comprised of endometrial epithelial regrowth, angiogenesis, and proliferation of endometrial stromal cells.

Cellular mechanisms of endometrial regeneration

Epithelial growth

After menstruation, the regeneration of all cell types, epithelial, endothelial, and stromal, occurs rapidly. The remaining basal layer acts as a germinal compartment from which the different cell types grow and differentiate [87]. Regrowth is estrogen dominated; and for epithelial cells EGF, TGFα, and EGF receptor are all likely to be involved. Both TGFα and EGF compete for the EGF receptor; and both, along

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 postmenstrual periods. VEGF-A levels are highest in the menstrual phase, presumably in response to proinflammatory 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

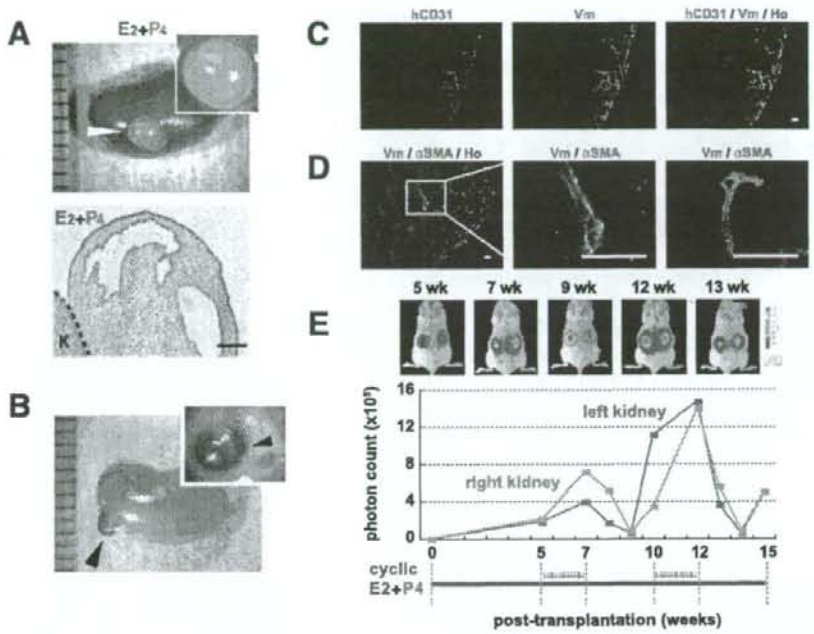

Fig. 3. Regeneration model of human endometrium in mice

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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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