those who did receive some antidepressant, the average dosage decreased to 45.1 (SD = 64.7) mg/day. All in all about 74% (70/84) of patients were prescribed no or inadequate drug doses, i.e. less than 75 mg/day. At maintenance phase immediately before relapse, 41% (12/29) were on no antidepressant drug. Among those who did receive some antidepressant, the average dosage was 42.0 (SD = 74.7) mg/day. Again 83% (24/29) were prescribed no or inadequate doses. Patients' refusal was the most common reason for not using antidepressant. None of the patients were administered systematic psychotherapy including cognitive-behaviour therapy or interpersonal therapy.

Discussion

We once reported on the treatment received by patients with depression for the acute phase treatment, and noted their undertreatment (Furukawa *et al.*, 2000b). The current study followed the same cohort and examined the adequacy of the treatment during continuation and maintenance phases. Again we had to note the gross undertreatment as over 80% of the cohort did not receive antidepressants at or above the recommended dose levels, including more than half the subjects who received no medication, during continuation and maintenance phases.

A number of available studies in the literature appear to agree on the quasi-universal inadequacy of continuation and maintenance treatment of major depression. In Finland, of some 200 patients who initiated treatment for their unipolar major depression, about half (49%) terminated treatment prematurely and did not go on to receive continuation treatment (Melartin *et al.*, 2005). The figure was very similar in the United States, as 42% discontinued antidepressant therapy during the first 30 days and only 28% continued antidepressant therapy for more than three months (Olfson *et al.*, 2006). In yet another study, only 32 of 99 patients (32%) received adequate continuation treatment (Kobak *et al.*, 2002). Ramana *et al.* (1999) in UK found that about 80% of patients were prescribed adequate dose of drugs, more than 125 mg/day, and this figure declined slowly but by 18 months approximately 43% were still being prescribed adequately and 40% received no antidepressant. In the Netherlands, 24% of antidepressant prescription for depression were shorter than a month, while 21% were longer than six months. Seventy-eight percent of these prescriptions were below the recommended effective dosage (van Weel-Baumgarten *et al.*, 2000).

Various factors have been found to be associated with inadequate continuation and maintenance treatments. Low education and low income predicted earlier termination, whereas poor pretreatment health status, treatment with new generation antidepressants and psychotherapy were associated with longer continuation treatment (Olfson *et al.*, 1996) History of antidepressant treatment and severer index episode predicted longer treatment (Melartin *et al.*, 2005). Physicians' failure to explicitly advise patients to continue on medication and patients' reluctance to discuss adverse effects with their physicians also predicted earlier discontinuation (Bull *et al.*, 2002).

The advice concerning the duration of treatment contained in the 1985 Consensus Development Conference Statement is probably still appropriate: Duration of the treatment must be determined on an individual basis depending on the previous pattern of episodes, degree of impairment produced, the adverse consequences of a new recurrence and the patient's ability to tolerate the drug. The National Institute of Mental Health consensus panel concluded that stronger the indications for initiating preventive treatment, the longer its duration should be.

Possible weaknesses of the present study may include our failure to collect information regarding several important factors in determining the adequacy of antidepressant treatment, including duration of antidepressant prescription and emergence of side effects. If we take the duration of antidepressant prescription into account, the proportion of patients receiving adequate antidepressant treatment would be even lower. Secondly, treatment was not controlled in this naturalistic study and therefore the level of treatment can be seen as either a cause or an effect. For instance, high levels of treatment are associated with a worse outcome or sicker subjects get more treatment. Thirdly, although the participating centres represented various treatment settings from all over Japan, it is safe to assume that they represent relatively motivated practitioners. The actual practices in less motivated clinical settings could be even more dismaying. Lastly, our findings relate mainly to the situation more than 10 years ago and to tricyclic antidepressants. The present day situation could be different, with more acceptable drugs and with stronger evidence of effectiveness of long-term continuation/maintenance treatments (Geddes *et al.*, 2003).

On the other hand, strengths of this study would include the following. The 23 study settings of the current study were fairly representative of psychiatric practices in Japan and the study sample was a random subset of all hospital admissions to these hospitals. Longitudinal interviewing for each month aided more accurate identification of onset and offset of periods of depression and subsyndromal symptomatology.

Conclusions

We confirmed that Japan was no exception to other industrialized countries in its provision of continuation and maintenance treatment to major depression. Although the majority of the patients diagnosed with major depression were administered some antidepressant, its dosage was very often below the recommended effective range even 1–6 months after commencement of the treatment. Newer drugs including SSRIs and SNRIs are now available. A further study of actually provided treatments in present day Japan would be of interest.

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REVIEW

Molecular and Cellular Mechanisms for Differentiation and Regeneration of the Uterine Endometrium

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Abstract. The human endometrium undergoes cyclical changes including proliferation, differentiation, tissue breakdown, and shedding (menstruation) throughout a woman's reproductive life. The postovulatory rise in ovarian progesterone induces profound remodeling and differentiation of the estradiol-primed endometrium. This change, termed decidualization, is crucial for embryo implantation and maintenance of the pregnancy. To date, activation and crosstalk of cAMP- and progesterone-mediated signaling pathways have emerged as key cellular events to drive integrated changes at both the transcriptome and the proteome levels. This results in the induction and maintenance of the decidual phenotype and function. Our recent series of studies highlights the critical role of SRC kinase activation (v-src sarcoma viral oncogene homolog) and STAT5 (signal transducer and activator of transcription 5) phosphorylation in decidualization. After separation of the functional layer of the differentiated endometrium that follows progesterone withdrawal, *i.e.*, menstruation, the basal layer of the endometrium, under the influence of estradiol, regrows and initiates a unique form of angiogenesis and regenerates a new functional layer. The molecular and cellular mechanisms for this process remain elusive, mainly because of difficulties in reproducing menstrual tissue breakdown, shedding, and subsequent tissue regeneration *in vitro*. We have recently developed a "humanized" mouse model in which a functional human endometrium is reconstituted. It may be used as an *in vivo* experimental tool for the study of endometrial angiogenesis and regeneration. This model may also be used to identify and test new therapeutic strategies for endometriosis, endometrial cancer, implantation failure, and infertility related to endometrial dysfunction.

Key words: Endometrium, Decidualization, Regeneration, SRC, Progesterone, Animal model

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THE maternal decidua intertwines with the invading fetal trophoblast at the fetomaternal interface, crucially regulating placental function and the growth and development of the conceptus. Decidualization can be defined as the postovulatory process of endometrial remodeling in preparation for pregnancy, which includes secretory changes of the uterine glands, differentiation of stromal cells, accumulation of specialized uterine natural killer cells, and vascular remodeling. In the humans, decidualization, which starts to take place in the luteal phase of the menstrual cycle, is more strictly defined. It denotes the progesterone-induced differentiation of fibroblast-like endometrial stromal cells (ESCs), located in the proliferative estrogen-primed endometrium, into decidual cells. Decidualization is characterized histologically by the appearance of larger and rounder cells surrounding the spiral arteries and eventually spreading through most of the endometrium. Following embryo implantation, decidualization persists and extends throughout the endometrium, leading to the formation of the pregnancy decidua. This morphological change is accompanied by integrated changes at both the transcriptome and the proteome levels. As a consequence, decidualizing ESCs acquire the unique ability to regulate trophoblast invasion, to resist inflammatory and oxidative insults, and to diminish local maternal immune responses. This process is accomplished through local production of numerous biological sub-

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stances including growth factors, cytokines, neuropeptides, free radical scavengers, and extracellular matrix components [1–6]. Major secretory products are PRL and IGF-binding protein 1 (IGFBP-1), two proteins that have been used widely as phenotypic markers of decidual cells [7]. Thus, decidualization is crucial for successful embryo implantation and maintenance of the pregnancy. Impaired decidual responses may cause a variety of endometrial and pregnancy disorders including infertility, recurrent miscarriages, uteroplacental dysfunction, endometriosis, and endometrial cancer.

ESCs isolated from human endometrium and cultured in the presence of progesterone in combination with cAMP or estradiol (E_2), exhibit morphological and functional changes *in vitro* that mimic *in vivo* decidual transformation [8]. With the development of this *in vitro* model of decidualization, many studies have addressed the molecular mechanisms underlying decidual transformation. Abundant clinical and experimental evidence substantiates that this differentiation process largely depends on the convergence of the progesterone and cAMP signaling pathways [8]. These pathways, alone, however, cannot completely account for functional and morphological characteristics of the decidual phenotype. Decidualized human ESCs (hESCs) produce many bioactive substances, including growth factors and cytokines, whose downstream signaling pathways also may contribute to decidual transformation in a paracrine/autocrine manner [1–3, 6].

The first half of this review summarizes the molecular mechanisms responsible for the initiation and maintenance of decidualization. The second half briefly addresses what is currently understood regarding the cellular mechanisms underlying endometrial regeneration and presents our recently developed experimental model for the study of the process.

Hormone signals for decidualization

Progesterone signaling

Progesterone and its receptor action

i. Genomic action

It is generally accepted that progesterone initiates and drives decidualization. In endometrial cells, progesterone exerts its actions predominantly through activation of the progesterone receptor (PR), which is a member of the nuclear receptor family of ligand-dependent transcription activators.

The general pathway of progesterone-inducible PR-mediated gene transcription is well characterized. Progesterone binding induces a conformational change(s) in the PR that promotes dissociation from a multi-protein chaperone complex. This is followed by homodimerization and binding to specific progesterone response elements (PREs) within the promoter of target genes [9]. Indeed, there are two putative progesterone responsive elements in the promoter region of IGFBP-1 [10], one of the representative decidual markers. DNA bound receptors increase rates of gene transcription by influencing recruitment of RNA polymerase II to the initiation site. Through protein-protein interactions, the hormone activated PR recruits coactivators that serve as essential intermediates for transmitting signals from the receptor to the transcription initiation complex. Coactivators facilitate transcription initiation through protein interactions with components of the general transcription machinery, and by promoting local remodeling of chromatin at specific promoters. Nuclear receptor-associated coactivators possess intrinsic enzyme activity for acetylation (histone acetyltransferase activity, HAT) or methylation of core histone proteins. These modifications of core histones relieve the repressive effects of chromatin on transcription and facilitate access of the general transcription machinery [11].

The PR is expressed as two different sized proteins from a single gene; PR-A and PR-B. PR-A lacks the 164 N-terminal amino acids of PR-B, is transcriptionally less active, and can transrepress PR-B on palindromic progesterone-responsive elements [12]. PR-A is the dominant isoform involved in decidualizing hESC *in vivo* and *in vitro*. In mice, PR-A null mice exhibit a defective decidual response to the implanting blastocyst [13–15]. In hESCs, the relative level of expression of these two PR isoforms may partially account for the escape of many decidual genes from the strict and direct transcriptional control of progestins. Furthermore, emerging evidence suggests a major role for the activated PR, specifically the PR-A isoform, as a scaffold for the both the direct and indirect recruitment of other activated transcription factors in response to cAMP signaling [8]. Direct physical interaction has indeed been demonstrated between the PR and the signal transducer and activator

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

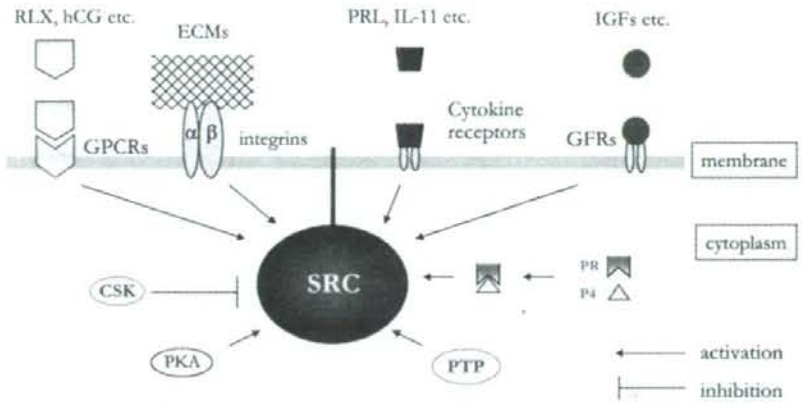

Fig. 1. Possible regulators of SRC activity in endometrial and decidual cells.
RLX, relaxin; hCG, human chorionic gonadotropin; GPCR, G protein-coupled receptors; ECMs, extracellular matrices; PRL, prolactin; IL-11, interleukin 11; IGFs, insulin-like growth factors; GFRs, growth factor receptors; CSK, 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-

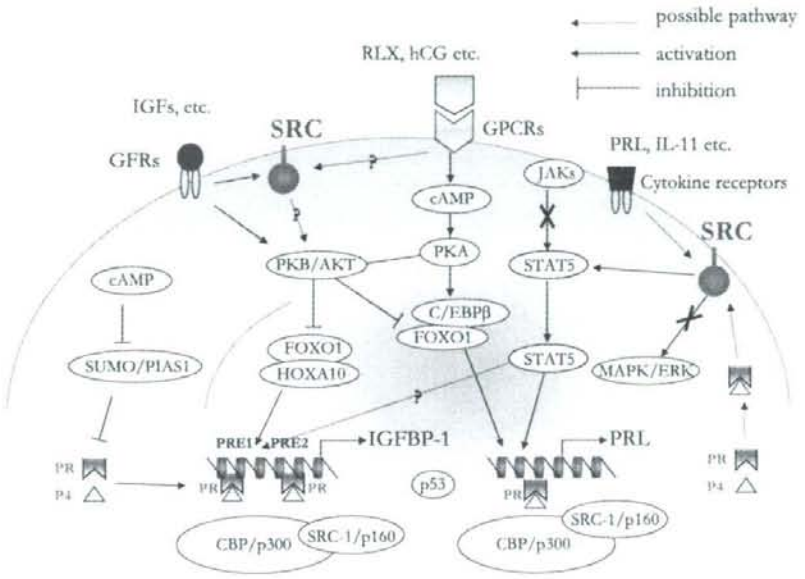

Fig. 2. Signaling events associated with decidualization.

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

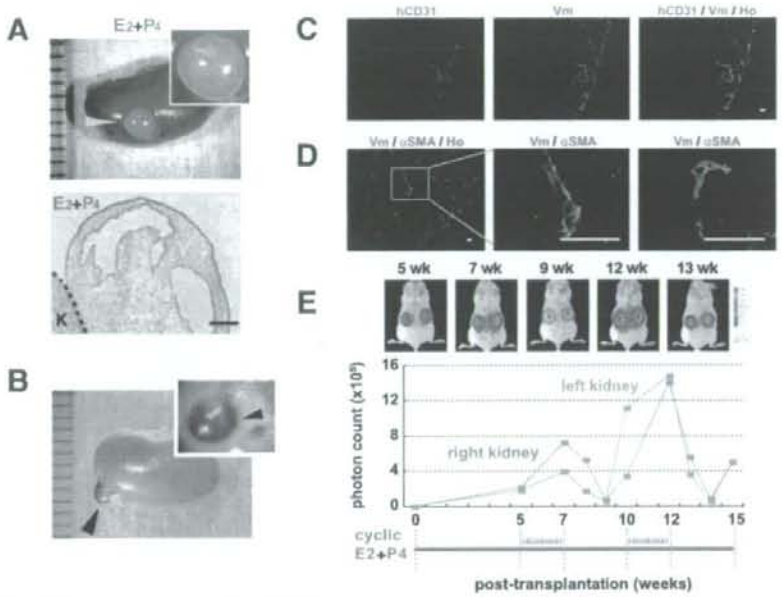

Fig. 3. Regeneration model of human endometrium in mice

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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Behavioral/Systems/Cognitive

Reverse of Age-Dependent Memory Impairment and Mitochondrial DNA Damage in Microglia by an Overexpression of Human Mitochondrial Transcription Factor A in Mice

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Mitochondrial DNA (mtDNA) is highly susceptible to injury induced by reactive oxygen species (ROS). During aging, mutations of mtDNA accumulate to induce dysfunction of the respiratory chain, resulting in the enhanced ROS production. Therefore, age-dependent memory impairment may result from oxidative stress derived from the respiratory chain. Mitochondrial transcription factor A (TFAM) is now known to have roles not only in the replication of mtDNA but also its maintenance. We herein report that an overexpression of TFAM in HeLa cells significantly inhibited rotenone-induced mitochondrial ROS generation and the subsequent NF-κB (nuclear factor-κB) nuclear translocation. Furthermore, TFAM transgenic (TG) mice exhibited a prominent amelioration of an age-dependent accumulation of lipid peroxidation products and a decline in the activities of complexes I and IV in the brain. In the aged TG mice, deficits of the motor learning memory, the working memory, and the hippocampal long-term potentiation (LTP) were also significantly improved. The expression level of interleukin-1β (IL-1β) and mtDNA damages, which were predominantly found in microglia, significantly decreased in the aged TG mice. The IL-1β amount markedly increased in the brain of the TG mice after treatment with lipopolysaccharide (LPS), whereas its mean amount was significantly lower than that of the LPS-treated aged wild-type mice. At the same time, an increased mtDNA damage in microglia and an impaired hippocampal LTP were also observed in the LPS-treated aged TG mice. Together, an overexpression of TFAM is therefore considered to ameliorate age-dependent impairment of the brain functions through the prevention of oxidative stress and mitochondrial dysfunctions in microglia.

Key words: mitochondria DNA; transcription factor A; oxidative stress; aging; memory impairment; microglia

Introduction

It is widely believed that oxidative stress and inflammation are major causative factors for a progressive decline in motor and memory functions during aging in humans and animals (Forster et al., 1996; Navarro et al., 2002). Behavioral dysfunctions associated with aging are also postulated to be associated with a decreased activity of mitochondrial electron transfer complexes with aging (Navarro et al., 2004, 2005). Furthermore, increased intracellular reactive oxygen species (ROS) activate microglia, which are representative resident mononuclear phagocyte populations in the brain, to induce an increased production of inflammatory mediators (Pawate et al., 2004; Qin et al., 2005). ROS generated in close proximity and in large concentrations by the mitochondrial respiratory chain cause oxidation of unsaturated fatty acid, proteins, and DNA. Mitochondrial DNA (mtDNA) is highly susceptible to damage produced by ROS because of its close proximity to ROS generation through the respiratory chain and its paucity of protective histones. Furthermore, there is little capacity for DNA repair in the mitochondria. During aging, a large number of mtDNA mutations accumulate in various tissues including the brain, thus leading to dysfunction of the respiratory

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chain. Finally, deficits in the respiratory chain result in the enhanced ROS production, culminating in age-dependent memory impairments (Corral-Debrinski et al., 1992; Lin et al., 2002). Therefore, the vulnerability of mtDNA to ROS is a major determinant factor for deficits in the brain functions in aging.

Mitochondrial transcription factor A (TFAM) is a nucleus-encoded protein that binds upstream of the light-strand and heat-strand promoters of mtDNA and promotes the transcription of mtDNA (Parisi and Clayton, 1991). Moreover, there is increasing evidence that TFAM plays an important role in maintaining mtDNA and regulating its copy number. The amount of mtDNA is closely correlated with the amount of TFAM but not with the transcription level, and the majority of TFAM molecules are involved in architecturally maintaining the higher structure of mtDNA (Kanki et al., 2004b). More recently, transgenic mice that overexpressed human TFAM under control of the β-actin promoter were generated and showed an increased copy number of mtDNA in the myocardium and the protection of the heart from mitochondrial dysfunction (Ikeuchi et al., 2005). The improvement in mitochondrial respiratory function may thus lead to an amelioration of the chronic process of remodeling by decreasing the mitochondrial ROS generation. The brain has a higher demand for oxygen; therefore, it is possible that increased oxidative stress and consequent mtDNA mutations may lead to the accumulation of lipid peroxidation products. Furthermore, it is reasonable to speculate that such TFAM overexpression may also inhibit the mitochondrial ROS generation through a reduction of the mtDNA mutations, which may retard the motor and memory functions.

To address this issue, the effects of TFAM overexpression on age-dependent deficits in brain functions were examined using human TFAM transgenic (TG) mice. The present study showed a significant improvement in the age-dependent memory impairments in TG mice because of a marked reduction in both oxidative stress and inflammation in the brain.

Materials and Methods

All experimental procedures of this study were approved by the Animal Care and Use Committee of Kyushu University.

Overexpression of TFAM in HeLa cells using the tetracycline-regulation system. Tetracycline-regulated TFAM-overexpressing cell lines were produced as previously reported (Parisi and Clayton, 1991). The cells were grown in DMEM containing 10% fetal bovine serum, 400 mg/ml Geneticin (G418), and 200 mg/ml hygromycin B, with or without 1 mg/ml doxycycline (DC) and maintained at 37°C in humidified air with 5% CO_2. The cells were then seeded in 24-well dishes and the culture medium was replaced after 24 h with serum-free DMEM containing 400 mg/ml G418 and 200 mg/ml hygromycin B, with or without 1 mg/ml DC. After 24 h, the cells were treated for 6 h with 0.001% of 1 mol/L rotenone dissolved in ethanol. The protein levels of human TFAM in the soluble fractions of HeLa cells in the presence and absence of DC were analyzed by immunoblotting.

Rotenone-induced ROS measurement. Intracellular ROS was measured by a ROS-sensitive fluorescent probe, 2,7-diamino-10-ethyl-9-phenyl-9,10-dihydrophenanthridine (DHE), in tetracycline-regulated HeLa cells. The cells were plated in 96-well culture dishes and cultured for 24 h at 37°C in humidified air with 5% CO_2, followed by incubation in serum-free medium for another 24 h. Next, the cells were treated with 1 μmol/L rotenone for 5.5 h and then with 2 μmol/L DHE for 30 min. The fluorescent intensity was determined immediately at excitation wavelength of 485 nm and emission wavelength of 530 nm on a fluorescent plate reader. The cellular images for DHE oxidation were observed using confocal laser-scanning microscope (CLSM) (LSM510MET; Carl Zeiss).

CLSM images for nuclear factor-κB nuclear translocation. Tetracycline-regulated TFAM-overexpressing HeLa cells were seeded in 24-well dish at a density of 10^3 cells/mm^2. The culture medium was replaced after 24 h with serum-free DMEM medium containing 400 mg/ml G418 and 200 mg/ml hygromycin B, with or without 1 mg/ml DC. After 24 h, the cells were treated for 6 h with 0.001% (final concentration in medium) of 1 mol/L rotenone dissolved in ethanol. After treatment with rotenone or vehicle, the cells were fixed with 4% paraformaldehyde and then incubated with mouse anti-p65 monoclonal antibody (Santa Cruz Biotechnology; AH Diagnostics) in bovine serum albumin (BSA)/PBS overnight at 4°C, washed three times with PBS, and then incubated with a secondary Alexa 488 goat anti-mouse IgG antibody in BSA/PBS for 1 h at room temperature. After three washes, the nuclei of cells were counterstained with propidium iodide (Sigma-Aldrich). The cells showing bright staining for p65 in the nucleus were scored, and the results were presented as a percentage of the number of cells with nuclear factor-κB (NF-κB) nuclear translocation to the total number of cells examined.

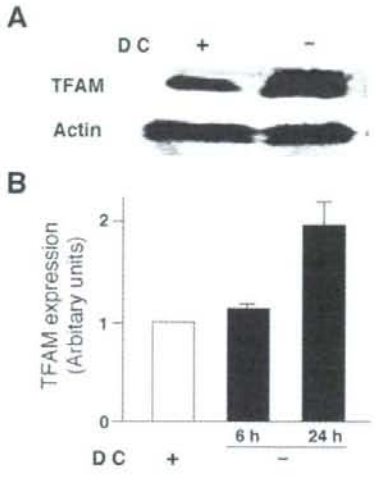

Figure 1. Protein levels of human TFAM overexpressed by the tetracycline-off system in HeLa cells. ***A***, Immunoblot analysis of human TFAM expressed in HeLa cells cultured with and without DC for 24 h. ***B***, The mean protein level of human TFAM in HeLa cells cultured with and without DC for 6 and 24 h. The mean relative immunoreactivity of each protein band was determined using the level of actin as an internal control. Each column and bar represent the mean and SEM of three experiments, respectively.

Animals. The methods for generating TG mice that overexpressed human TFAM has been described previously (Ikeuchi et al., 2005). The animals were housed under 12 h light/dark cycle (lights on at 8:00 A.M.) with access to food and water *ad libitum*. All mice were handled daily for 5 d before the start of the experiment to minimize stress reactions to manipulation.

Immunoblotting. Antibodies against human TFAM and mouse Tfam were produced by immunizing rabbits with recombinant glutathione *S*-transferase-tagged human TFAM and mouse Tfam. The protein levels of human TFAM and mouse endogenous TFAM were analyzed in the soluble fractions of brain tissue homogenates as previously described (Lin et al., 2002).

Thiobarbituric acid reactive substances. Twenty male C57BL/6 mice [wild type (WT) and TG] of the following age groups: young (2 months of age; WT, $n = 5$; TG, $n = 5$) and aged (24 months of age; WT, $n = 5$; TG, $n = 5$) were used for the measurement of thiobarbituric acid reactive substances (TBARS). The homogenates of whole brains were mixed with 0.4% SDS, 7.5% acetic acid adjusted to pH 3.5 with NaOH, and 0.3% thiobarbituric acid. The mixture was kept at 5°C for 60 min and then heated at 100°C for 60 min. After cooling, the mixture was extracted with distilled water and *n*-butanol:pyridine (15:1, v/v) and centrifuged at 16,000× *g* for 10 min. The fluorescence of the supernatant was measured at excitation and emission wavelengths of 510 and 550 nm, respectively, using GENios Pro (Tecan). The standard was prepared using TEP (1,1,3,3-tetraethoxypropane).