—Full Paper—

Involvement of Stathmin in Proliferation and Differentiation of Immortalized Human Endometrial Stromal Cells

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Abstract. Uterine endometrial stromal cells differentiate into decidual cells during the late secretory phase of the menstrual cycle and pregnancy. However, the biochemical mechanisms of decidualization have yet to be definitively elucidated. In the present study, we transfected primary human endometrial stromal cell with a temperature-sensitive mutant of simian virus 40 large T antigen and thereby established an immortalized stromal cell line (EtsT) in order to examine the role of stathmin, a cytosolic phosphoprotein that regulates microtubule dynamics, in stromal cell differentiation. When treated with the decidual stimulus dibutyryl-cAMP (db-cAMP) or forskolin, the fibroblastic cell-shaped EtsT cells transformed into large- and round-shaped cells and secreted large amounts of the decidual markers prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1). Analysis of the stathmin protein levels in the db-cAMP- and forskolin-treated EtsT cells revealed that the total and phosphorylated protein levels dropped as decidualization progressed. Suppression of stathmin expression by transfection with small interfering RNA (siRNA) suppressed EtsT cell proliferation. It also abolished db-cAMP-induced PRL and IGFBP-1 mRNA expression and protein secretion. Thus, stathmin expression can be considered an integral factor regulating the initial stage of the process of human endometrial stromal cell differentiation.

Key words: A microtubule regulatory protein, Decidualization, Endometrium, Human, Stathmin
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In humans and ruminants, approximately 10–15% of all pregnancies end in spontaneous abortion, mostly because of implantation failure [1]. Implantation failure is thought to result from failure of embryos to develop and/or failure of the uterus to prepare itself for pregnancy during the peri-implantation period. In the latter case, stromal cell decidualization is an essential event that prepares the uterus for blastocyst implantation and

thus the establishment of pregnancy. To date, many factors that are critical for successful implantation have been demonstrated, and the biological activities or functions in the establishment of pregnancy have already been characterized [2, 3]. Nevertheless, the precise cellular and molecular mechanisms governing conceptus implantation, uterine decidualization, and placental formation have not been definitively elucidated; this imposes a major limitation on diagnosis and treatment of pregnancy disorders.

Stathmin (Op18) is a ubiquitous cytosolic

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phosphoprotein that has four serine residues at its N-terminus [4, 5]. Our recent study demonstrated that stathmin is one of several genes whose expression is specifically upregulated in the rat uterus at the site of embryo attachment and at decidual formation during the decidualization process [6, 7]. This suggests that stathmin may be associated with decidualization of endometrial stromal cells in rodents. Stathmin is also highly expressed in the nervous system and by several cancers, including ovarian and breast cancers [5]. Furthermore, stathmin gene family mRNA levels are particularly high in reproductive tissues, including the uterus in humans [8].

Stathmin functions as a regulator of microtubule (MT) dynamics during cell cycle progression by promoting MT disassembly [10, 11]. It thus increases MT turnover and thereby aids rapid reorganization of the MT cytoskeleton. It also promotes the catastrophes of tubulin by binding to MT plus ends, thereby stimulating the exposed β -subunit of the terminal tubulin dimers to hydrolyze guanosine 5'-triphosphate (GTP) [11]. Extensive phosphorylation of stathmin by various kinases has been shown to turn off its MT-destabilizing activity, which in turn promotes tubulin polymerization [12, 13].

To date, the differentiation of stromal cells has largely been studied by using primary cultured stromal cells. These types of studies have shown that when uterine endometrial stromal cells transform into decidualized stromal cells, they are altered morphologically and begin to secrete specific decidual proteins [14]. *In vitro* studies have also shown that stromal cell preparations decidualize when exposed to ovarian steroids (progesterone and estradiol) or agents that activate the cAMP pathway [15]. In our previous report, we also used primary endometrial stromal cell cultures to explore the role of stathmin in decidualization. However, this type of *in vitro* research is hampered by limitations in the number of primary stromal cells that can be obtained from human patients; more importantly, the differentiation potential of these primary cells is maintained for only a short period. We established an immortalized endometrial stromal cell line to overcome these limitations and employed it to examine the physiological role of stathmin during the *in vitro* decidualization process.

Materials and Methods

Tissue collection and immortalization of endometrial stromal cells

Endometrial stromal cells were prepared from the endometrial tissues of patients undergoing surgery or biopsies, as described previously [16]. Sample collection was conducted with the informed consent of the patients in accordance with the requirements of the clinical research ethics committee of the Tokyo Medical University Hospital. Normal tissue samples at the proliferative stage were minced finely with scissors and washed in phosphate buffered saline. The endometrial stromal cells were then isolated as described previously [17]. Briefly, the minced tissue samples were digested in calcium- and magnesium-free Hank's solution (CMF-Hanks) containing 0.25% collagenase (Type IA; Sigma-Aldrich, St. Louis, MO, USA) and then strained through a 250- μ m stainless steel sieve (Sanpo, Tokyo, Japan) to remove undigested tissue and mucous material. To remove glandular cells, the filtrates were passed through a 38- μ m sieve (Sanpo) through which stromal cells can pass. The passed solutions were centrifuged and resuspended in a small volume of CMF-Hanks. These cell preparations were at least 95% positive for vimentin, and they showed no staining for cytokeratin. For culture, the stromal cells were resuspended in basal medium (DMEM/F12 without phenol red; Life Technologies, Tokyo, Japan) containing 50 U/ml penicillin, 50 μ g/ml streptomycin, 0.25 μ g/ml fungizone (Life Technologies), and 10% (v/v) charcoal-treated fetal bovine serum (stripped FBS; HyClone, South Logan, UT, USA) and seeded in a tissue culture flask (25 mm², Asahi Techno Glass, Tokyo, Japan). The medium was removed 2 h later, and the adherent cells were washed with fresh medium and cultured to 40% confluency for 5 days.

The cells were then infected for 16 h with retrovirus particles in polybrene that produced a temperature-sensitive form of the simian virus 40 large T antigen (SV40tsT) and were cultured at the permissive temperature of 32 C until continuously growing cells appeared, as described previously [18]. The retrovirus was produced using PLAT-E retrovirus-packaging cells and the retroviral vector pMESVts, which carries the SV40tsT and neomycin acetyltransferase genes. After removing the medium and washing the cells, culture of the cells

was continued for about 2 months for cell cloning. Four clones that had high proliferation rates were defined as EtsT endometrial stromal cell lines. One of these lines, denoted as EtsT-ZD1, was used in the present study.

Experimental schedule

EtsT cells were passaged into 12-well culture plates with basal medium containing 10% stripped FBS and antibiotics and cultured for about 2 days at 32 C for growth to subconfluency. To induce decidualization, the cells were cultured for 6 days at 39 C in basal medium (1 ml/well) containing 2% stripped FBS and antibiotics supplemented with 1 mM dibutyryl cyclic AMP (db-cAMP) or 25 mM forskolin. The medium was changed daily and collected 3 and 6 days after decidualization was stimulated. The culture media were examined for the presence of IGF-binding protein-1 (IGFBP-1) and PRL, which are well-known decidual markers. The cultured cells were subjected to immunoblotting (see below) or semi-quantitative RT-PCR analyses (see below). To suppress stathmin expression, the stromal cells were pretreated with stathmin siRNA 24 h before stimulating decidualization (see below).

Immunoblot analysis of IGFBP-1, PRL, stathmin, and phosphorylated stathmin expression

The cells grown in the culture dishes were lysed using Chaps Cell Extract Buffer (Cell Signaling Technology, Beverly, MA, USA) according to the manufacturer's instructions. The culture medium (10 μ g protein) or cell lysate (3 μ g protein) was then subjected to 15–20% gradient SDS-polyacrylamide gel electrophoresis (PAGE) for analysis of IGFBP-1, PRL, and stathmin expression as described previously [16]. We used a native PAGE system that separates stathmin phosphoisomers according to their charge differences to detect phosphorylated stathmin. After electrotransfer onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), the membranes were blocked with Blocking Buffer (Pierce, Rockford, IL, USA). Immunoblot analysis was performed using an rabbit anti-IGFBP-1 antiserum (1:1,000; Upstate Biotechnology, Lake Placid, NY, USA), anti-PRL antibody (1:1000; SAMP#518, anti-hPRL-IC-5, National Hormone and Peptide Program), anti-stathmin serum (1:10,000; kindly donated by A. Sobel, Institut du Fer a Moulin, Paris, France), and anti-phospho-

rylated stathmin antibody (1:1,000; p-Op18: ser16, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoreactive bands were detected by enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA, USA) after incubation with horseradish peroxidase-labeled goat anti-rabbit IgG antibody (0.5 μ g/ml, Vector Laboratories, Burlingame, CA, USA). As a loading control, the same membrane was re-probed with anti- β -actin antibody (1:1,000; Sigma-Aldrich). All blotting experiments were repeated at least three times, and representative data are shown.

RNA isolation and RT-PCR analysis

Poly(A)⁺ RNA was isolated using a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). The poly(A)⁺ RNA (0.25 μ g) was then reverse-transcribed and amplified using a One Step RNA PCR Kit (TaKaRa, Ohtsu, Japan) according to the manufacturer's instructions. The sense (S) and antisense (AS) primers used are as follows: 5'-GTCGCTTGCTCTTCTATTACACC-3'(S), 5'-ATGGGGAGAAAGTCAGTTCTC-3' (AS) for stathmin; 5'-TGCTGCAGAGGGAGGGAGCCCC-3'(S), 5'-AGG GATCCTCCTTCCCATTCCA-3'(AS) for IGFBP-1; 5'-GACAGAGACACCAAGAAGAATCGGAAC ATA-3'(S), 5'-GCAATGGAACGGATCATTAAG GACCTTCTC-3'(AS) for PRL; and 5'-ACCACA GTCCATGCCATCAC-3'(S), 5'-TCCACCACCCTG TTGCTGTA-3' (AS) for glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The G3PDH primers served as an internal control. The PCR protocols used were as follows: 20 cycles of 95 C for 1 min, 55 C for 45 sec, and 72 C for 2 min. The amplified products were separated on 1.5% agarose gels containing 0.45 μ g/ml ethidium bromide.

Treatment with siRNA

EtsT cells at 60% confluency were transfected with irrelevant control siRNA (50 nM; Santa Cruz Biotechnology) or stathmin siRNA (50 nM; Santa Cruz Biotechnology) for 24 h according to the manufacturer's instructions. After siRNA treatment, the cells were incubated with or without db-cAMP (1 mM) in basal medium supplemented with 2% stripped FBS at 37 C for 6 days.

Cell proliferation assay

To determine the effect of suppression of stathmin on the proliferative capacity of EtsT cells,

Fig. 1. Effect of decidual stimulation on EtsT cell morphology and IGFBP-1 and prolactin (PRL) secretion. EtsT cells were cultured for 6 days in the presence of 1 mM db-cAMP or 25 mM forskolin. **A:** Morphology of EtsT cells on day 6 of culture. Photographs were taken under a phase contrast microscope. Bars=100 μ m. **B:** Day 0, 3 and 6 culture media containing 10 μ g protein were subjected to immunoblot analysis for IGFBP-1 and PRL. The numbers at the top of Figure 1B indicate the day of culture.

cell proliferation was determined by WST-1 assay (Cell Counting Kit, Dojindo, Kumamoto, Japan). Briefly, EtsT cells (4×10^4) were seeded in 24-well culture plates in DMEM/F12 medium supplemented with 10% stripped FBS, transfected with siRNA 24 h later, and cultured for another 24 or 48 h. The cells harvested at these time points were incubated with WST-1 reagent for 20 min at 37 C. The staining intensity in the medium was measured by absorbance at 450 nm, and the data were expressed as ratios of the control value.

Statistical analysis

The cell proliferation rate data were presented as means $\pm$ SEM. Statistical significance ($P < 0.05$) was determined using the Dunnett test.

Results

Ability of db-cAMP or forskolin to induce EtsT decidualization

The immortalized endometrial stromal cells that we established in our laboratory were designated as EtsT cells. After 6 days of culture in culture

medium containing 2% FBS, these cells exhibited a spindle-shaped morphology (Fig. 1A, control), which is similar to primary stromal cells [16]. To determine whether EtsT cells retain the characteristics of primary endometrial stromal cells, we examined their ability to decidualize upon stimulation with forskolin (an activator of protein kinase A) or the cAMP analog db-cAMP. In the presence of either stimulator, the fibroblastic cell-shaped EtsT cells changed morphologically into larger- and round-shaped cells (Fig. 1A, middle and right panels). This morphological change was observed two days after decidual stimulation (data not shown).

To evaluate whether these morphological changes were associated with appropriate functional changes, we examined whether IGFBP-1 and PRL secretion into the culture medium was enhanced. While the unstimulated cells produced barely detectable amounts of IGFBP-1 and PRL throughout days 0–6, the db-cAMP- and forskolin-treated cells clearly secreted more of these markers on days 3 and 6 of culture (Fig. 1B). Notably, the PRL band appeared to be more intense with the db-cAMP treatment than with forskolin and was more intense on day 6 than on day 3 after either decidual stimulation.

Stathmin expression and phosphorylation during EtsT cell decidualization

We next investigated how the expression of stathmin protein levels changed during EtsT decidualization (Fig. 2). Thus, EtsT cells were treated with db-cAMP or forskolin, and their total stathmin protein (A) and phosphorylated stathmin (B) levels were determined by immunoblot analysis. The total stathmin protein levels of the untreated cells tended to decrease over time, while the stathmin form with three or four phosphorylated sites almost completely disappeared by days 3 and 6 of culture. However, these effects were much more pronounced in the db-cAMP- and forskolin-stimulated cells. Indeed, total and phosphorylated stathmin expression had almost completely disappeared in these cells by day 6 of culture.

Effects of stathmin suppression on EtsT cell proliferation

The effect of stathmin suppression on EtsT proliferation was evaluated using the WST-1 assay (Fig. 3). As expected, the stathmin siRNA-transfected cells had reduced stathmin levels 24 h

Fig. 2. Stathmin and phosphorylated stathmin levels during forskolin- or db-cAMP-induced EtsT cell decidualization. The cells were cultured for 6 days with 1 mM db-cAMP or 25 mM forskolin with daily media changes. Cell lysates were prepared on days 0, 3 and 6 of culture, and lysates containing 3 μ g protein were separated using SDS polyacrylamide gels (upper panel) or glycerol/urea gels (middle panel) and subjected to immunoblot analysis using a polyclonal anti-stathmin antibody (upper panel) or anti-phosphorylated stathmin (Ser-16) antibody (middle panel). The same membrane was re-probed with an anti- β -actin as a loading control (lower panel).

and 48 h after transfection (Fig. 3A). Importantly, the cells also had significantly lower proliferation rates 48 h after siRNA treatment compared with the control cells; smaller reductions in proliferation rates were observed at 24 h (Fig. 3B).

Effects of stathmin suppression on EtsT decidualization

To determine the physiological significance of stathmin in *in vitro* decidualization, we examined the effect of stathmin mRNA suppression on db-cAMP-induced EtsT decidualization (Fig. 4). Thus, EtsT cells were pretreated with stathmin siRNA for 24 h prior to db-cAMP stimulation. The siRNA treatment markedly decreased stathmin mRNA expression, with minimal expression observed on days 3 and 6 (Fig. 4A); this inhibitory effect lasted until at least day 6 and was similar to what has been observed with stathmin siRNA-treated, freshly prepared primary stromal cells [16]. Significantly, the stathmin siRNA also suppressed db-cAMP-stimulated IGFBP-1 and PRL transcription (Fig. 4A). Moreover, immunoblot analysis of the culture media revealed that siRNA treatment markedly inhibited db-cAMP-stimulated secretion of both the IGFBP-1 and PRL proteins (Fig. 4B). The cellular stathmin and IGFBP-1 levels were also markedly

Fig. 3. Effect of stathmin suppression on EtsT cell proliferation. The cells were treated for 24 h with stathmin siRNA or an irrelevant control siRNA (stathmin siRNA: -) and then further cultured for 24 and 48 h. A: The stathmin levels in the EtsT cells were determined by Western blot analysis to confirm stathmin suppression. The membrane was re-probed with β -actin as a loading control. B: The proliferation rates of the cells were determined using WST-1 reagent. The data are represented as ratios of the control and are shown as means $\pm$ SEM from three independent experiments. * $P < 0.01$ vs. stathmin siRNA (-).

inhibited by siRNA treatment (data not shown). However, there were no differences in the morphological changes to larger and rounder phenotype cells between the control and stathmin siRNA-treated groups, as shown in Fig. 4C.

Discussion

In the present study, we established a human EtsT cell line and showed that it has cAMP-sensitive differentiation potential. We demonstrated that siRNA-induced downregulation of stathmin expression decreased the proliferation of EtsT cells. We then showed that suppression of stathmin

Fig. 4. Effect of stathmin suppression on db-cAMP-induced EtsT cell decidualization. The cells were transfected with stathmin siRNA 24 h before decidual stimulation and then cultured for 6 days in the absence (–) or presence (+) of 1 mM db-cAMP. The culture media and cells were collected on days 0, 3 and 6 of culture. **A:** Total EtsT cell RNA was subjected to RT-PCR analysis for IGFBP-1, PRL and stathmin expression. G3PDH served as an internal control. **B:** The IGFBP-1 and PRL levels in the culture media were determined by immunoblot analysis. **C:** Phase contrast microscopy images of control and stathmin siRNA-treated cells. The cells transfected with control siRNA or stathmin siRNA were cultured for 3 days in the presence of 1 mM db-cAMP and then photographed. Bars=100 μ m.

expression blocked db-cAMP-induced decidualization of the EtsT cells. Thus, our study supports previous observations suggesting that stathmin may be involved in the proliferation and/or differentiation of primary endometrial cells in humans [16].

Ovarian steroids induce endometrial stromal cell differentiation by activating the cAMP pathways

and stromal cells that are freshly isolated from normal endometrial tissues decidualize when treated with either progesterone plus estradiol or db-cAMP [15]. In fact, several endometrial cell lines respond to steroid treatment [20]; however, the EtsT cell line does not decidualize upon treatment with progesterone plus estradiol (data not shown). Similarly, an endometrial cell line established in another laboratory did not respond to progesterone plus estradiol [21]. Thus, it appears that the intracellular signaling system between progesterone receptor (PR) activation and cAMP generation may be impaired in EtsT cells. Furthermore, this line may have lost its functional PR during establishment.

Stathmin expression is upregulated specifically at the site of embryo implantation and in the decidual zone but is downregulated as decidualization progresses in rodents [6, 7]. In humans, a significant increase in stathmin expression is not observed at the onset of decidualization. EtsT proliferation decreased in this study when endogenous stathmin mRNA expression was suppressed before decidual stimulation. This suggests that stathmin may regulate stromal cell proliferation prior to decidualization. On the other hand, it has been proposed that stathmin may aid by limiting “overproliferation” of cells during the cell cycle prior to cell commitment and/or differentiation [4]. Thus, stathmin probably promotes or maintains proper proliferation and possibly negatively downregulates excessive proliferation. Importantly, our observations of decreased decidual marker (IGFBP-1 and PRL) expressions following stathmin mRNA suppression suggest that stathmin is likely involved in preparation for or initiation of decidualization. Unexpectedly, suppression of stathmin mRNA expression did not affect the morphological changes usually seen after decidual stimulation. This result may indicate that the morphological changes during decidualization processes are not necessarily associated with enhanced secretion of IGFBP-1 and PRL. These results suggest that stathmin is probably involved in changes in secretory function during decidualization.

We also found that decidual stimuli induced the EtsT endometrial stromal cell line to differentiate and that this was associated with a decrease in stathmin expression over time. Moreover, we

found that EtsT decidualization was associated with acute decreases in the levels of the phosphorylated forms of stathmin. These observations are basically consistent with the changes in stathmin levels detected in primary endometrial stromal cell cultures undergoing decidualization [16]. Thus, it appears that as endometrial stromal cells begin to differentiate, stathmin activity drops. Given that stathmin promotes MT destabilization and is downregulated by its sequential phosphorylation, it is likely that the drop in stathmin activity permits the formation of the MT network and allows the mitotic spindle to form. This is supported by the fact that phosphorylated stathmin levels increase as cells enter the G2/M phase of the cell cycle. Indeed, phosphorylation of stathmin at multiple sites is required for orderly progression of the cell cycle [5]. Thus, both phosphorylation (inactivation) and dephosphorylation (reactivation) of stathmin are essential for cytokinesis and proper progression of the cell cycle. The decrease in both the total and phosphorylated stathmin levels of the stromal cells is consistent with a previous study showing that stathmin is highly expressed in undifferentiated cells but decreased during the induction of cell differentiation [22, 23]. Thus, it appears that the function of stathmin in decidualization is no longer required as endometrial stromal cells begin to differentiate. Stathmin levels then drop, thereby permitting formation of the MT network and promotion of stromal cell differentiation into decidual cells.

The decidualization of endometrial stromal cells that occurs in the late secretory phase of the menstrual cycle is characterized by morphological and functional changes [3, 24] that involve exit from the cell cycle. Thus, regulation of endometrial stathmin expression could be associated with the changes in the cell cycle in the endometrium during the secretory phase. The observation that the promoter region of the stathmin gene contains three E2F-binding sites suggests that stathmin expression and activity are linked to the cell cycle because DNA replication and mitotic activity are regulated through E2F [25]. Since cyclin D promotes E2F-mediated transactivation by releasing active E2F from pRB [26], it is possible that stathmin expression is regulated indirectly by cyclin D3 and p27^{kip1}. Indeed, p27^{kip1} has been found to impair stathmin function, thereby

inhibiting cell motility [27, 28]. The precise role stathmin plays in decidualization is unclear. However, it is possible that its MT destabilizing activity may facilitate important changes in the cytoskeleton that support subsequent differentiation-related changes in endometrial stromal cells. This is supported by the fact that differentiation of stromal cells is associated with cytoskeletal changes [29]. For example, in the baboon endometrium, the actin cytoskeleton is regulated by interactions between integrins and the extracellular matrix [30]. Moreover, the cytoskeleton of stromal cells is reorganized by the interleukin 1 β secreted by embryos, which enhances cyclooxygenase and matrix metalloproteinase-3 activity in stromal cells; these activities dissociate the cytoskeleton, including α -smooth muscle actin, and induce decidualization. Furthermore, disruption of the actin filament by cytochalasin D promotes stromal cell decidualization [31]. Significantly, it has recently been shown that both hCG and db-cAMP can prevent stromal cell apoptosis after cytoskeletal disruption *in vitro* [32]. Thus, decidualization appears to require disruption of the cytoskeleton. While it has not yet been shown whether stathmin expression can regulate actin filament dynamics, it is possible that the MT instability induced by increased stathmin expression facilitates disruption of the cytoskeletal apparatus and thereby promotes stromal cell decidualization.

In conclusion, the clear suppression of decidualization in EtsT cells subjected to suppression of stathmin expression prior to decidual stimulation observed in the present study indicates that stathmin plays a role during the initial stage of endometrial stromal cell decidualization.

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