Expression of stathmin, a microtubule regulatory protein, is associated with the migration and differentiation of cultured early trophoblasts

Mikihiro Yoshie1, Hideaki Kashima1, Tosio Bessho2, Makoto Takeichi2, Keiichi Isaka3 and Kazuhiro Tamura1,4

1Department of Endocrine Pharmacology, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan; 2Yoneyama Maternity Hospital, 2-12 Shinchou, Hachioji, Tokyo 192-0065, Japan; 3Department of Obstetrics and Gynecology, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

4Correspondence address. Tel/Fax: +81-42-676-4536; E-mail: hiro@ps.toyaku.ac.jp

BACKGROUND: The microtubule-destabilizing protein stathmin is expressed by the villous cytotrophoblasts and invasive extravillous trophoblasts (EVTs) in the first-trimester human placenta. Here, we evaluated the significance of stathmin expression in terms of the functions of trophoblasts. METHODS: We employed two choriocarcinoma cell lines (BeWo and JEG-3), an EVT cell line (HTR-8/SVneo) and isolated first-trimester trophoblast cells. The effects of small-interfering (si) RNA-mediated stathmin knockdown on trophoblast proliferation and migration were measured by WST-1 and Transwell assays, respectively. Trophoblast differentiation was induced by dibutyryl (db)-cAMP treatment and evaluated by measuring human chorionic gonadotrophin β (hCGβ) and syncytin expression and cell fusion. We examined the effect of knockdown and induced stathmin expression on db-cAMP-induced differentiation. RESULTS: siRNA-induced silencing of stathmin expression had a marked inhibitory effect on BeWo, JEG-3 and HTR-8/SVneo cell migration and also suppressed their proliferation, albeit to a lesser extent. db-cAMP-enhanced hCGβ and syncytin expression and cell fusion in BeWo cells was inhibited by stathmin knockdown. However, induced expression of stathmin reversed the hCGβ and syncytin expression and cell fusion in the Tet-On BeWo cells. Suppression of stathmin expression also inhibited the migration of and hCGβ production by first-trimester trophoblasts. CONCLUSIONS: Stathmin expression may be closely associated with early trophoblast migration and differentiation into syncytiotrophoblasts during placentation.

Keywords: stathmin; trophoblast; placenta; syncytiotrophoblast; extravillous trophoblast

Introduction

The placenta is a unique organ that plays a major role in establishing the physiological relationship between mother and fetus. In early pregnancy, mononuclear cytotrophoblasts fuse and differentiate into a continuous layer of multinucleated syncytiotrophoblasts that cover the floating chorionic villi. The syncytiotrophoblasts produce high levels of hormones such as hCG, placental lactogen and progesterone, which are indispensable for the continued progression of pregnancy. During the functional differentiation of trophoblasts, trophoblasts also express syncytin, which is a membrane protein that originates from the envelope gene of an endogenous retrovirus. This protein appears to play a key role in the fusion of villous cytotrophoblasts that lead to syncytiotrophoblast formation (syncytialization) (Frendo et al., 2003). The process that leads to syncytiotrophoblast differentiation remains imperfectly understood, although it is known that a cAMP analogue (Feinman et al., 1986), forskolin (Wice et al., 1990) and epidermal growth factor (Morrish et al., 1987; Amemiya et al., 1994) can each induce the in vitro differentiation of primary cytotrophoblasts and choriocarcinoma cell lines into syncytiotrophoblasts.

Other cells that are involved in early pregnancy events are extravillous trophoblasts (EVTs), which develop from anchoring villi. EVTs are different from syncytiotrophoblasts in terms of their invasion and migration abilities. While the precise molecular causes of pre-eclampsia and fetal growth restriction remain unknown, it is believed inadequate EVTs invasion and inappropriate remodelling of the uterine spiral arterioles may be involved (Lim et al., 1997).

Stathmin (Op18) is a cytosolic phosphoprotein that contains four serine phosphorylation sites at its N-terminus (Sobel and Tashjian, 1983; Hailat et al., 1990). This intracellular protein is abundant in the central nervous system and parts of the
reproductive tracts, including the uterus and ovary, and is highly evolutionarily conserved among the vertebrates (Koppel et al., 1990; Bieche et al., 2003). The main functions of stathmin are to regulate microtubule dynamics, which is mediated by its ability to interact with tubulin (Belmont and Mitchison, 1996; Belmont et al., 1996; Curmi et al., 1997), and cell cycle progression (Luo et al., 1994; Marklund et al., 1994; Larsson et al., 1995; Mistry and Atweh, 2001). We recently showed that stathmin is highly expressed by rodent placental tissues (Yoshie et al., 2004) and that stathmin protein is expressed in first-trimester human placentas by villous cytotrophoblasts and EVTs but not syncytiotrophoblasts (Tamura et al., 2006). No obvious signals were observed in the term placentas. This differential expression of stathmin by particular trophoblast cell types suggests that stathmin may be involved in the formation of the placenta. To determine the physiological significance of stathmin expression by human trophoblasts, we here examined the effect on trophoblast functions of knocking down stathmin expression.

Materials and Methods

Trophoblast isolation and culture

The choriocarcinoma cell lines, BeWo and JEG-3 cells, were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown at 37°C in Ham’s F-12 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin and 0.25 μg/ml amphotericin B. The EVT cell line HTR-8/SVneo was kindly provided by Dr Charles Graham (Queen’s University, Kingston, ON, Canada) and cultured with RPMI 1640 medium supplemented with 5% FBS and the antibodies/antimycotic mentioned above. Placental tissues at 6–10 weeks of pregnancy were totally obtained with informed consent from 15 women undergoing legal miscarriage. Among the 15 tissue samples, we used 8 for the migration assay (Fig. 2D), 3 for the matrix metalloproteinase (MMP) activity assay (Fig. 2F) and 4 for reverse transcription (RT)–PCR and immunoblot analysis (Fig. 3B and D). In each experiment, one tissue or pooled sample from two to three tissues was used. The use of these tissues in the experiments described herein was approved by the Clinical Research Ethics Committee of the Tokyo University of Pharmacy and Life Sciences (Permission No. 0702). The villous placentas were thoroughly washed with Ca2+/Mg2+-free Hanks’ solution to remove the blood, after which they were minced and incubated with 0.25% trypsin (Sigma-Aldrich, St Louis, MO, USA) and 0.25% collagenase Type IA (Sigma-Aldrich) for 20 min at 37°C in a water bath with continuous gentle shaking. The dispersed cells were strained through a 250 μm sterilized sieve (Sanpo, Tokyo, Japan) to remove undigested and mucosal tissues. Trophoblasts were collected from the filtrates by using Percoll gradient fractionation as described previously (Bischof et al., 1995), after which they were cultured with Ham’s F-12 medium containing 15% (v/v) FBS and antibiotics/antimycotic. The purity of trophoblasts was confirmed by immunostaining of cytokeratin and vimentin. More than 98% of the isolated trophoblast cells were positive for cytokeratin and negative for vimentin.

Treatment with siRNA

The BeWo, JEG-3, HTR-8/SVneo and isolated first-trimester trophoblast cells with 60% confluency in 12-well culture plates were transfected with non-targeting control small-interfering (si) RNA (50 nM; Qiagen, Mississauga, ON, Canada) or stathmin siRNA (50 nM; Santa Cruz Biotechnology, Santa Cruz, CA, USA) using LipofectamineRNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. After treatment for 24 h with siRNA, the medium containing siRNA and transfection reagents was removed, and after washing, the cells were cultured for 24–72 h with fresh culture medium.

Cell proliferation assay

To determine the effect of stathmin suppression on trophoblast proliferation, we used a tetrazolium reagent, 2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-1, Cell Counting Kit, Dojindo, Kumamoto, Japan). This assay is based on the measurement of the Formazan dye that is liberated after the cleavage of this reagent by mitochondrial dehydrogenase activity in viable cells. Briefly, BeWo, JEG-3 and HTR-8/SVneo cells (5 × 103 cells/well) were seeded in 48-well culture plates in their basal media supplemented with 10% FBS, transfected with stathmin or control siRNA 24 h later and cultured for another 24–72 h. The cells were then incubated with WST-1 reagent for 20 min at 37°C. The staining intensity in the medium was measured by determining the absorbance at 450 nm, and the data were expressed as ratios of the control value.

Migration assay

Trophoblast migration was carried out using the Transwell system (Chemotaxicell; Kurabo, Osaka, Japan) equipped with 8-μm pore size polycarbonate filters. Cells transfected with control or stathmin siRNA were trypsinized, resuspended in their basal media containing 2% FBS and loaded onto the fibronectin-coated upper compartment. The chambers were then placed into 24-well culture plates containing the basal media supplemented with 10% FBS to enhance their migration. After 48 or 72 h of incubation, the non-invading cells remaining in the upper compartment were removed using cotton swabs and the cells that had migrated past the lower surface of the filters were fixed with 4% paraformaldehyde and stained with 4’,6-diamidino-2-phenylindoe, dihydrochloride (DAPI, Invitrogen). For each experiment, the number of cells in five randomly chosen fields of each filter was counted. The results of three independent experiments are presented as the mean ± standard deviation.

Gelatin zymography

The activities of MMP-2 and -9 in the culture medium were semi-quantitatively evaluated by gelatin zymography after trophoblast treatment with stathmin siRNA for 48 h. Briefly, the media were collected and clarified by centrifugation to remove cells and debris. Samples (25 μg protein) were then loaded under non-reducing conditions onto SDS–polyacrylamide (7.5%) gel containing 0.25 mg/ml gelatin. Following electrophoresis, the gels were washed with 2.5% (v/v) Triton X-100 to remove the SDS, after which they were incubated in a developing buffer (50 mM Tris–HCl (pH 7.5), 10 mM CaCl2 and 150 mM NaCl) for 15 h at 37°C. The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA) and then destained in the same solution without dye. Gelatinase activity was visualized as white bands on the blue-stained gelatin background.

Induction of trophoblast differentiation

BeWo and first-trimester trophoblast cells were incubated with or without the cAMP analogue dibutyryl (db)-cAMP (1 mM; Sigma-Aldrich) in Ham’s F-12 medium supplemented with 1% FBS at...
37 °C for 3 days to induce enhanced hCG secretion and cell fusion, which are indicators of cell differentiation. The medium was replaced every day.

**RNA isolation and semi-quantitative RT–PCR analysis**

Total RNA was extracted by using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer’s procedure. The total RNA (0.25 μg) was reverse-transcribed and amplified by using a One Step RNA PCR Kit (TaKaRa, Shiga, Japan). The RT–PCR procedure for stathmin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been described previously (Tamura et al., 2006). To amplify human chorionic gonadotropin β (hCGβ) and syncytin, the following sense (S) and antisense (AS) primers were used: 5'-TGT TGC TGC TGC TGA GCA TG-3' (S), 5'-CAG AGT GCA CAT TGA CAG CTG-3' (AS) for hCGβ, and 5'-CAT ACC TCA AAC CTC ACC TG-3' (S), 5'-GTT GAC CTT GCA AGG TGA CC-3' (AS) for syncytin. Amplification was performed over 20 cycles of 94°C for 30 s, 56°C for 45 s and 72°C for 2 min. The amplified products were separated on 1.5% agarose gels containing 0.45 μg/ml ethidium bromide. The intensity of the bands was analysed using Scion Image software (Scion Corp., Maryland, MD, USA), and each value was normalized against that of the GAPDH band in corresponding lane.

**Immunoblot analysis of stathmin and hCGβ expression**

The harvested culture medium was immediately stored at −30°C until hCGβ immunoblot analysis could be performed. The cells were lysed with Chaps Cell Extract Buffer (Cell Signaling Technology, Beverly, MA, USA). The culture media (3 μg protein) and cell lysates (2 μg protein) were subjected to 10–20% gradient SDS–polyacrylamide gel electrophoresis, and blotting membranes were prepared. The membranes were incubated with rabbit antibodies against stathmin as detailed previously (Tamura et al., 2007) and mouse antibodies against hCGβ (1:500; AbD Serotec, Oxford, UK). The immunoreactive bands were detected by enhanced chemiluminescence (PerkinElmer Life Science, Wellesley, MA, USA) after incubation with horseradish peroxidase labelled mouse or rabbit IgG antibody (0.5 μg/ml; Vector Laboratories, Burlingame, CA, USA). The membrane was re-probed with mouse anti-β-actin antibody (1:10 000; Sigma-Aldrich) as a loading control. All blotting experiments were repeated at least three times, and representative data are shown.

**Cell fusion assay**

Cells fusion (syncytialization) is a unique morphological change in trophoblast differentiation. BeWo cells were stained with anti-desmosomal protein antibody (clone ZK-31; Sigma-Ardich) along with an Alexa Fluor 594-labeled goat anti-mouse IgG (Invitrogen) to distinguish cell border. The nuclei were counterstained with DAPI. The number of multinuclear cells in the five areas under microscope which were randomly selected was counted. The data are expressed as the ratio of each control, and the effect of stathmin siRNA on db-cAMP-induced cell fusion was evaluated from four independent experiments.

**Generation of Tet-On inducible BeWo cells**

The Tet-On gene expression system (Clontech, PaloAlto, CA, USA) was used to examine the effect of induced stathmin expression on BeWo cell differentiation. Briefly, by using FuGENE HD transfection reagent (Roche, Indianapolis, IN, USA), BeWo cells (1 × 10^6 cells/10-mm dish) were transfected with 10 μg of pTet-On Advanced vector, which expresses the doxycycline (Dox)-controlled fusion protein of a reverse tetracycline transactivator (rtTA) and the C-terminal domain of protein 16 of herpes simplex virus (VP16). The neomycin-resistant clones were then selected and screened by luciferase assays for low background and high inducibility in response to Dox. The clone showing the highest rtTA activity was selected and named Tet-On BeWo.

The open-reading frame of the human stathmin gene was amplified by PCR and subcloned into pTRE-Tight vector, which contains a human CMV promoter with heptamerized Tet operators. This promoter is silent in the absence of rtTA binding to the Tet operators. However, when the reverse Tet repressor of rtTA binds to the operators, the VP16 domain of rtTA can activate CMV activity to a very high level and switch on expression of the target gene (stathmin in this case). BeWo Tet-On cells were transiently transfected with pTRE-stathmin to induce stathmin expression. Cells transfected with pTRE-tight served as controls. The pTRE-stathmin-transfected cells were stimulated with Dox (1 μg/ml; Clontech) to induce stathmin expression. The effect of induced stathmin expression on BeWo cell differentiation was examined after endogenous stathmin was knocked down.

**Statistical analysis**

Each experiment was repeated at least two times (see each figure’s legend) and the results were reproducible. The results of cell proliferation and migration are presented as means ± SEM. The data of cell fusion and the densitometrical analyses of gene expression are shown as means ± SD. Statistical analysis was examined by ANOVA with post hoc comparison of Dunnett’s test, and P < 0.05 values were considered as statistically significant.

**Results**

**Knockdown of stathmin represses trophoblast proliferation**

To examine the effect of knocking down stathmin on trophoblast proliferation, the choriocarcinoma cell lines BeWo and JEG-3 and the EVT cell line HTR-8/SVneo were transfected with non-targeting control or stathmin siRNA. We confirmed that stathmin siRNA repressed endogenous stathmin expression in these cell lines for at least 48 h (data not shown). All three lines showed a significant decrease in cell proliferation after stathmin siRNA transfection (Fig. 1). This was most evident at the 48 h time point after transfection, where the proliferation of BeWo, JEG-3 and HTR-8/SVneo cells was decreased by 50.4 ± 4.9, 32.4 ± 6.0 and 31.3 ± 6.2%, respectively.

**Stathmin knockdown inhibits trophoblast migration**

To determine whether stathmin is associated with trophoblast migration, we used a Transwell assay to assess the effect of suppressing stathmin expression on trophoblast migration. Stathmin siRNA treatment inhibited the migration of BeWo, JEG-3 and HTR-8/SVneo cells by 43.0 ± 14.7, 61.0 ± 6.4 and 46.7 ± 5.4%, respectively (Fig. 2A–C). It also suppressed the migration of primary trophoblasts isolated from first-trimester placentas by 45.9 ± 8.5% (Fig. 2D).

It has been reported that MMP-2 and -9 participate in trophoblast invasion by degrading the extracellular matrix (ECM) of the maternal decidua (Cohen et al., 2006). To determine whether stathmin expression affects the production of active MMPs by trophoblasts, we used gelatin zymography to measure the MMP-2 and -9 activities in the conditioned medium of the stathmin siRNA-treated trophoblasts. However, stathmin siRNA treatment did not change MMP-2 or -9 secretion.
Stathmin knockdown represses db-cAMP-induced trophoblast differentiation

The differentiation of cytotrophoblasts into syncytiotrophoblasts is essential for the maintenance of a normal pregnancy. When exposed to cAMP analogue 8-bromo-cAMP, cultured trophoblasts differentiate into syncytiotrophoblasts that show enhanced expression of hCGβ and the cell fusion marker syncytin (Frendo et al., 2003). To determine whether stathmin expression is associated with db-cAMP-induced differentiation, the effect of stathmin knockdown on db-cAMP-induced hCGβ and syncytin expression was evaluated in BeWo and first-trimester trophoblasts. As expected, stathmin siRNA treatment inhibited stathmin mRNA expression in BeWo cells in the absence or presence of db-cAMP. Moreover, db-cAMP elevated the levels of hCGβ and syncytin mRNA in control siRNA-treated BeWo cells. However, when stathmin expression was knocked down, the db-cAMP-induced expression of hCGβ and syncytin mRNA was suppressed (Fig. 3A). With regard to first-trimester trophoblasts, stathmin siRNA treatment suppressed db-cAMP-stimulated hCGβ expression, but not syncytin expression (Fig. 3B). Immunoblot analysis of the conditioned media of BeWo and first-trimester trophoblasts confirmed that stathmin siRNA repressed their db-cAMP-induced hCGβ secretion (Fig. 3C and D). Further, we examined the effect of stathmin knockdown on db-cAMP-induced cell fusion of BeWo cells. In control siRNA transfected cells, db-cAMP increased the number of multinuclear cells that showed cell fusion, whereas in stathmin siRNA-treated cells, db-cAMP-induced cell fusion was significantly repressed (Fig. 3E and F). Our previous study revealed that differentiated syncytiotrophoblasts in human first-trimester villi show very low levels of stathmin expression (Tamura et al., 2006). Thus, we examined how stathmin expression in normal BeWo cells changes during their db-cAMP-induced differentiation. Indeed, db-cAMP treatment of normal BeWo cells decreased their stathmin expression at both the mRNA and protein levels (Fig. 3A, B, G and H). Thus, endogenous stathmin expression is down-regulated during trophoblast differentiation.

Enforced vector-derived stathmin expression makes stathmin siRNA-treated cells susceptible to db-cAMP-induced differentiation

To further examine the role that stathmin plays in BeWo cell differentiation, Tet-on BeWo cells were treated with control or stathmin siRNA, transiently transfected with pTRE-stathmin vector and then cultured with or without Dox (1 μg/ml), which induces stathmin expression from the pTRE-stathmin vector. Two days later, the cells were treated with db-cAMP and cultured for another 3 days, after which the hCGβ and syncytin mRNA expression levels were evaluated by semi-quantitative RT–PCR analysis. In the absence of Dox treatment, stathmin siRNA treatment decreased db-cAMP-induced hCGβ and syncytin expression (Fig. 4A and B), as shown in the result of Fig. 3A and B. In other words, BeWo cells that have low expression of stathmin fail to differentiate fully upon db-cAMP stimulation. However, when Dox treatment forced vector-derived stathmin expression in these cells, they expressed hCGβ and syncytin at appropriate levels upon db-cAMP treatment (Fig. 4A and B). In stathmin siRNA transfected cells, Dox-mediated induced expression of stathmin recovered db-cAMP-induced hCGβ secretion (Fig. 4C). Dox treatment had no effect on the db-cAMP-induced hCGβ and syncytin mRNA expression in control siRNA-treated cells (data not shown). The db-cAMP-induced cell fusion of Tet-On BeWo cells was examined (Fig. 4D and E). In the absence of Dox, stathmin siRNA treatment reduced the db-cAMP-stimulated cell fusion compared with control siRNA group [siRNA (−)/Dox (−)]. When stathmin expression was recovered by Dox treatment in stathmin siRNA-treated cells, the number of db-cAMP-mediated cell fusion significantly increased up to the control level.

Discussion

The establishment of a successful pregnancy requires that, at the early stage of placentation, trophoblasts acquire the ability to migrate, invade and secrete hormones. We have shown here that when stathmin expression was knocked down, BeWo, JEG-3, HTR-8/SVneo and first-trimester trophoblast cell migration was partial and significantly suppressed, as compared to control. It should be noted that stathmin knockdown not only
affected the migration of the above cells, it also suppressed their proliferation except first-trimester trophoblasts. However, in JEG-3 and HTR-8/SVneo cells, stathmin knockdown inhibited their migration much more severely than their proliferation. This suggests that the ability of stathmin siRNA to inhibit trophoblast migration is not merely due to its ability to suppress cell proliferation. Notably, first-trimester trophoblasts, which are a heterogeneous cell population containing endovascular and interstitial EVTs, villous cytotrophoblasts and syncytiotrophoblasts migrated less well than the other three types of trophoblasts that we tested. The cells that migrated from the primary trophoblast population on the upper chamber of the Transwell system were probably invasive EVTs. Ozon et al. (2002) were the first to report that a decrease in stathmin expression impairs germ cell migration in Drosophila. Other studies using a mouse endothelial cell line (Miyashita et al., 2004), rat neural cells (Jin et al., 2004; Giampietro et al., 2005), mouse sarcoma cells (Baldassarre

Figure 2: Effect of stathmin knockdown on the migration and MMP activities of trophoblasts.

(A–D) BeWo (A), JEG-3 (B), HTR-8/SVneo (C) cells and isolated first-trimester placenta trophoblasts (D) were transfected with control (−) or stathmin (+) siRNA for 24 h, re-plated onto fibronectin-coated transwell chambers and incubated for 48 h (HTR-8/Svneo) or 72 h (other trophoblasts). The cells that penetrated to the opposite side of the membrane were stained with DAPI, and those in five randomly chosen fields were counted. The upper panels show representative photographs of DAPI-stained migrated cells. The lower panels depict the mean ± SEM of the migration ratios (relative to the control value) determined in three independent experiments. *p < 0.05, **p < 0.01 versus siRNA (−). (E, F) BeWo, JEG-3, HTR-8/SVneo (E) and first-trimester trophoblasts (F) were cultured for 96 h after transfection with control or stathmin siRNAs and the cultured media were subjected to gelatin zymography to detect MMP-2 and -9 activities. Representative data from three experiments are shown.
et al., 2005) and human umbilical vein endothelial cells (Mistry et al., 2007) subsequently also revealed that stathmin may be involved in cell migration. Moreover, embryonic fibroblast cells from stathmin-deficient mice migrate more slowly than those from wild-type animals, and this phenotype is rescued by inducing stathmin expression (Baldassarre et al., 2005).

The latter study also showed that a cyclin-dependent kinase inhibitor, cytosolic p27kip, represses migration by binding to...
stathmin and inactivating the microtubule-depolymerization activity of stathmin. These reports support our hypothesis that stathmin knockdown-induced inhibition of trophoblast migration is due to changes in microtubule dynamics. Given the above-mentioned link between p27kip and stathmin in trophoblast migration (Baldassarre et al., 2005), it is of interest that EVTs and villous cytotrophoblasts express p27kip (Korgun et al., 2006). Also of interest is that Nodal, a member of the transforming growth factor β superfamily, and its receptor, activin receptor-like kinase 7 (ALK7), are also expressed by human placenta. When the Nodal–ALK7 pathway is activated, it inhibits proliferation, in part by increasing p27kip expression (Roberts et al., 2003; Munir et al., 2004). Thus, it is possible that stathmin may participate in trophoblast motility by interacting with p27kip.

During the process of embryo implantation, EVTs invade into the maternal decidua and the deeper portion of the myometrium by degrading the ECM. The invasive property of trophoblasts correlates closely with their ability to secrete proteases, in particular MMPs (Cohen et al., 2006). MMP-2 and -9 are expressed by human first-trimester placenta trophoblasts, and the elevation of their MMP-9 expression correlates closely with the time of trophoblast invasion (Xu et al., 2000). However, we found that stathmin siRNA treatment had no effect on the ability of trophoblasts to produce active MMP-2 or -9. Thus, the silencing of stathmin expression partially inhibits trophoblast migration without decreasing their MMP activities.

When trophoblasts are treated in vitro with db-cAMP, they differentiate into syncytiotrophoblasts. We have shown here that stathmin expression decreased when BeWo and

**Figure 4:** Effect of induced stathmin expression on the db-cAMP-induced differentiation of BeWo cells with low stathmin expression. (A–C) Tet-On BeWo cells that stably express rtTA were treated with control (−) or stathmin (+) siRNA for 24 h and transiently transfected with pTRE-stathmin vector. The clones that expressed stathmin in response to Dox treatment were treated with 1 μg/ml Dox to stimulate stathmin expression. Two days later, 1 mM db-cAMP was added and the cells were cultured for another 3 days. (A) Total RNA was subjected to semi-quantitative RT–PCR to determine hCGβ, syncytin, stathmin and GAPDH expression. (B) Densitometrical analyses of stathmin, hCGβ and syncytin normalized with GAPDH level. (C) The culture media were subjected to immunoblotting using an anti-hCGβ antibody. (D, E) The stathmin expression in Tet-On BeWo cells that were transfected with the siRNAs was recovered by Dox treatment. The cells were then cultured for 2 days with db-cAMP and stained with DAPI (blue) and anti-desmosomal protein (red) to indicate cell fusion. Representative pictures are shown and the multinuclear cells are marked with dotted lines (D). The number of multinuclear cells in five randomly selected areas was counted. The data are represented as ratios of each control [E: siRNA(−)/Dox (−)]. The data from two independent experiments are shown as mean ± SD. (n = 4).
first-trimester trophoblasts were treated with db-cAMP. This is consistent with our previous observation showing that stathmin localizes in vivo in first-trimester placental tissue in the EVTs and villous cytotrophoblasts but not the syncytiotrophoblasts (Tamura et al., 2006). Moreover, Nampoothiri et al. (2007) have shown by proteomic profiling analysis that the stathmin gene is down-regulated in differentiated BeWo cells. Similarly, stathmin expression is decreased during the process of human endometrial stromal cell differentiation (decidualization) (Tamura et al., 2006, 2007). Another novel finding of this study is that while knocking down stathmin represses the mRNA expression and protein secretion of hCGβ in both BeWo and first-trimester trophoblast cells, these effects are reversed by the induced expression of stathmin (at least in BeWo cells). When BeWo cells were treated with db-cAMP, syncytin expression increased and this effect was repressed by stathmin siRNA treatment. Furthermore, we showed that knocking down stathmin suppressed the db-cAMP-induced cell fusion of BeWo cells while induced expression of stathmin recovered their cell fusion activity. These observations suggest that stathmin expression may influence trophoblast differentiation.

Notably, db-cAMP and/or stathmin siRNA treatment did not alter syncytin expression. In fact, the trophoblasts isolated from first-trimester placentas did not respond to db-cAMP in terms of their syncytin expression (Fig. 3B). It has been shown previously that BeWo and normal trophoblast cells differ in their gene expression profiles (García and Castrillo, 2004). Furthermore, although hCG secretion is enhanced during trophoblast differentiation, it has been established that hCG secretion and syncytialization are not necessarily directly linked (Taylor et al., 1991) and that hCG-producing capacity does not parallel the degree of syncytialization. Moreover, it was reported recently that syncytin is expressed by a variety of trophoblast cells, including villous trophoblasts and EVTs (Malassine et al., 2005; Muir et al., 2006). Thus, syncytin expression is also not a reliable marker of syncytialization. Consequently, we evaluated the loss of intracellular boundaries by using desmosome staining to show that stathmin is involved in syncytialization.

Although the mechanism by which stathmin regulates trophoblast differentiation remains unknown, it is likely that stathmin is involved in the preparation or initiation of trophoblast differentiation. This notion is based on our observation that trophoblast differentiation was significantly suppressed when stathmin expression was knocked down by siRNA treatment prior to receiving the db-cAMP stimulus. In particular, it is likely that stathmin-mediated modulation of microtubule dynamics participates in trophoblast differentiation. Supporting this is the study by Douglas and King (1993), who demonstrated that the microtubule-disrupting drug colchicine dose-dependently inhibits hCGβ secretion and the formation of multinucleated syncytiotrophoblasts by cultured cytotrophoblast cells. Thus, knocking down stathmin may stabilize the microtubule network and thereby inhibit trophoblast differentiation. It should be noted that stathmin knockout mice do not suffer from any fertility defects (Schubart et al., 1996), which might be seen as an argument against the possibility that stathmin participates in trophoblast function. However, stathmin is a generic member of the intracellular phosphoprotein family that contains a number of proteins that are highly conserved in vertebrates, including SCG10, SCG10-like protein (SCLIP), RB3 and its splice variants RB3α and RB3β (Ozon S et al., 1997; Charbaut et al., 2001). These family members all possess a tubulin-interacting stathmin-like domain in their C-termini along with various N-terminal extensions containing cysteine residues that serve as palmitoylation sites with they associate with intracellular membranes (Di Paolo et al., 1997; Curmi et al., 1999). The concomitant presence of these members of the intracellular phosphoprotein family may compensate for the loss of stathmin in the stathmin knockout mice.

In conclusion, we showed by using in vitro trophoblast migration and differentiation models that stathmin expression affects in the cytotoxic trophoblast migration and differentiation that is accompanied by hCGβ synthesis and syncytialization. These multiple roles of stathmin in trophoblasts suggest that stathmin may be an essential regulator of placentation in early pregnancy.

**Funding**

This work was partially supported by a Grant-in-Aid for Scientific Research (19790763 to M.Y.) from the Japan Society for the Promotion of Science and by the High-Tech Research Centre Project for Private Universities and Initiatives for Attractive Education in Graduate Schools from the Ministry of Education, Culture, Sport, Science and Technology.

**References**


Submitted on October 16, 2007; resubmitted on July 22, 2008; accepted on July 24, 2008.