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Possible role of the exchange protein directly activated by cyclic AMP (Epac) in the cyclic AMP-dependent functional differentiation and syncytialization of human placental BeWo cells

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BACKGROUND: The mononuclear villous cytotrophoblast (CTB) differentiates and fuses to the multinucleated syncytiotrophoblast (STB), which produces hCG and progesterone. cAMP-mediated intracellular pathways are involved in the process of endocrine differentiation and fusion (syncytialization). The exchange protein directly activated by cAMP (Epac) is a mediator of cAMP signaling. We examined the differential roles of Epac and protein kinase A (PKA) signaling in the cell fusion and differentiation of trophoblast-derived BeWo cells.

METHODS: Epac1 and Epac2 were localized in human placental tissue ($n = 9$) by immunohistochemistry. The PKA-selective cAMP analog (N^6-phenyl-cAMP, Phe) or Epac-selective cAMP analog (CPT) was tested for effects on hCG and progesterone production, and syncytialization in BeWo cells. The effect of knockdown of Epac or its downstream target molecule (Rap1) on syncytialization was evaluated.

RESULTS: Epac1 and Epac2 proteins were expressed in villous CTB, STB, stroma, blood vessels and extravillous CTB of the placenta. Phe increased the expression of *hCGα/β* mRNA and secretion of hCG protein in BeWo cells ($P < 0.01$ versus control). CPT-stimulated production of hCG ($P < 0.05$), albeit to a lesser extent than Phe. Progesterone production was also enhanced by Phe or CPT ($P < 0.01$ and $P < 0.05$, respectively). CPT or a stable cAMP analog (dibutyryl-cAMP: Db) increased the number of syncytialized BeWo cells ($P < 0.01$), whereas Phe did not stimulate fusion. CPT- or Db-induced syncytialization was observed, even in the presence of a PKA inhibitor. Knockdown of Epac1 or Rap1 repressed the Db-, CPT- or forskolin-induced cell fusion.

CONCLUSIONS: The Epac signaling pathway may be associated with the cAMP-mediated functional differentiation and syncytialization of human trophoblasts.

Key words: Epac / syncytialization / hCG / progesterone / BeWo

Introduction

The placenta is a transiently formed multifunctional organ that is mainly composed of the fetal chorion and endometrial decidua. The major role of the placenta is to establish a crosstalk between the maternal and fetal circulations, which permits exchanges of gas and nutrition that are necessary for fetal development. In addition, the placenta functions as an endocrine tissue that produces various steroids, peptide hormones, growth factors and cytokines, which are crucial for the establishment and maintenance of pregnancy. The multinucleated syncytiotrophoblast (STB) layer, which covers floating villi in the placenta, is the interface cell layer that separates fetal and maternal blood (Benirschke and Kaufmann, 2000). The formation of the multinucleated STB layer via cell fusion of mononuclear cytotrophoblast (CTB) is referred to as syncytialization, which is a well-characterized morphological sign of their terminal differentiation. As STB lacks the ability of DNA replication, the layer is maintained by continuous CTB–CTB or CTB–STB cell fusion and apoptosis (Huppertz and Kingdom, 2004). The insufficient development of trophoblast syncytialization may cause pathological conditions, such as pre-eclampsia

(Gauster et al., 2008). The STB layer functions not only as a barrier between maternal and fetal blood vessels, but also as endocrine cells that produce hCG, progesterone and placental lactogen to maintain pregnancy and fetal development (Sullivan, 2004). hCG is a glycoprotein that is composed of an α subunit and a β subunit. The α subunit of hCG is similar to that of LH, FSH and thyroid-stimulating hormone. The specific action of hCG is dependent on the production capacity of hCGα of the placenta. The serum level of hCG increases rapidly after implantation and peaks at 8–10 weeks of human gestation. Progesterone is secreted from luteinized ovaries via hCG stimulation during early pregnancy; however, luteal progesterone secretion decreases with the reduction of hCG production. The STB layer functions as the main source of progesterone during pregnancy and the synthesis of progesterone increases gradually, up to the term of pregnancy (Tuckey, 2005).

It was reported that the enhancement of intracellular cAMP levels via treatment with various cAMP analogs (Chou, 1980; Lambot et al., 2005) and forskolin (FSK), which is an activator of adenylate cyclase (Nulsen et al., 1998), results in activation of protein kinase A (PKA) and promotes functional differentiation, such as the production of hCG and progesterone and syncytialization in primary isolated trophoblasts and in the trophoblast-derived human choriocarcinoma cell line BeWo.

The exchange protein directly activated by cAMP (Epac) was identified as a cAMP-mediated signaling factor that acts in a way that is different from the classical PKA signaling pathway (de Rooij et al., 1998; Kawasaki et al., 1998). There are two isoforms of Epac, Epac 1 (also known as cAMP-GEF-I) and Epac2 (cAMP-GEF-II), which are the products of different genes in mammals but share extensive sequence homology. Epac1 is expressed in all tissues, whereas Epac2 has a more limited distribution in the brain and adrenal glands (Kawasaki et al., 1998). Epac1 and Epac2 function as guanine-nucleotide exchange factors for the Ras family of small GTPases, Rap1 and Rap2 (Bos, 2006). The binding of cAMP to their cyclic nucleotide-binding domain causes a conformational change that promotes the recruitment of the Rap protein to the CDC25 homology domain and exchange of the GDP-binding inactivated form of Rap to the GTP-binding activated form of Rap. The study of Epac in cAMP-mediated physiological function has been accelerated by the synthesis of an Epac-selective cAMP analog and a PKA-selective cAMP analog, to distinguish these two cAMP signals. Epac-mediated cAMP signaling is involved in cellular functions such as cell differentiation, secretion/exocytosis, cell adhesion and cell–cell junctions (Bos, 2006; Roscioni et al., 2008). The importance of PKA-mediated cAMP signaling in placental formation is known (Keryer et al., 1998) but the physiological significance of the expression of Epac on trophoblast function remains unknown. In the present study, we determined the expression of Epac in the different stages of human placenta and investigated the possible association of Epac with functional differentiation and syncytialization.

Materials and Methods

Reagents

The Epac-selective cAMP analog [8-(4-chlorophenyltio)-2′-*O*-methyl cAMP, CPT] and the PKA-selective cAMP analog (N^6-phenyl-cAMP, Phe) were purchased from the Biolog Life Science Institute (Bremen, Germany). Dibutyryl-cAMP (Db) and FSK were from Sigma-Aldrich (St. Louis, MO, USA). All cAMP analogs were dissolved in water (50 mM stock solution). H89, which is a PKA inhibitor, was purchased from the D. Western Therapeutics Institute, Inc. (Nagoya, Japan) and dissolved in dimethylsulphoxide (10 mM stock solution, Wako Pure Chemical Industries Ltd., Osaka, Japan).

Tissue samples and cell culture

Normal placental tissues of first trimester (6 and 7 weeks of gestation; $n = 2$ each) and third trimester (37 and 38 weeks of gestation; $n = 2$ each) were obtained with informed consent from women undergoing surgery, such as Cesarean section. The use of these tissues in the experiments was approved by the clinical research ethics committee of the Tokyo University of Pharmacy and Life Sciences. The human choriocarcinoma cell line BeWo was purchased from the American Type Culture Collection (Manassas, VA, USA) and grown at 37°C in Ham's F-12/Dulbecco's modified Eagle's medium (1:1) medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin and 0.25 μg/ml amphotericin B.

RNA isolation and RT–PCR analysis

Total RNA was extracted using Isogen (Nippon Gene, Tokyo Japan), according to the manufacturer's instructions. The total RNA (100 ng) was subjected to real-time RT–PCR using the iScript One-Step RT–PCR Kit with SYBR Green (Bio-Rad Laboratories, Hercules, CA, USA). The reactions were carried out on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories). The sense (S) and antisense (AS) primers used for real-time RT–PCR were as follows: 5′-TCCCACTC CACTAAGGTCCAA-3′ (S), 5′-CCCCATTACTGTGACCCTGTT-3′ (AS) for *hCGα*; 5′-GCTACTGCCCCACCATGACC-3′ (S), 5′-ATGGAC TCGAAGCGCACATC-3′ (AS) for *hCGβ*; 5′-CACCTTCACCATGTCC AGAA-3′ (S), 5′-ATAAACCGACTCCACGTTGC-3′ (AS) for *P450scc (P450 side-chain cleavage enzyme)* and 5′-AGCCACATCGCTCAGACA-3′ (S), 5′-GCCCAATACGACCAAATCC-3′ (AS) for glyceraldehydes-3-phosphate dehydrogenase (*GAPDH)*. The fold change in expression of each gene was calculated using the ΔΔCt method using *GAPDH* as an internal control.

Immunoblotting

The harvested culture medium from BeWo cells was immediately stored at −30°C until hCGβ immunoblotting could be performed. Cells were lysed with Chaps Cell Extract Buffer (Cell Signaling Technology, Inc., Beverly, MA, USA), according to the manufacturer's instructions. Equal amounts of medium or lysate protein were subjected to sodium dodecyl-sulfate (SDS)-polyacryl-amide gel electrophoresis and electrophoretically transferred onto polyvinylidene difluoride membranes. The membranes were incubated with antibodies against hCGβ (clone INN-hCG-22, 1:500; AbD Serotec, Oxford, UK), Epac1 (catalog No. ab21235, 1:500; Abcam, Cambridge, MA, USA), Epac2 (clone 5B1, 1:1000; Cell Signaling Technology) or Rap1 (catalog No. 07-916, 1:1000; Upstate biotechnology, Lake Placid, NY, USA) at room temperature for 2 h. Immunoreactive bands were detected using enhanced chemiluminescence (PerkinElmer Life Science, Inc., Boston, MA, USA) after incubation with horseradish peroxidase-labeled goat anti-mouse or rabbit immunoglobulin (Ig)G antibodies (0.5 μg/ml, Vector Laboratories, Burlingame, CA, USA). The membrane was treated with a stripping solution [25 mM glycine–HCl, pH 2.0, containing 1% (w/v) SDS] and re-probed with antibody against GAPDH (clone GAPDH-71.1, 1:5000; Sigma-Aldrich). The relative band intensity of hCG was assessed by densitometric analysis of digitalized

autographic images using the Scion Image software (Scion Corp., Fredrick, MD, USA) and normalized to the amount of total cell protein.

Progesterone assay

The concentration of progesterone in the culture medium was measured by radioimmunoassay (RIA), as described previously (Tamura *et al.*, 1991) and normalized to the amount of total cell protein.

Treatment with small interfering RNA

BeWo cells grown in 24-well culture plates to ~60% confluency were transfected with a non-targeting control small interfering RNAs (siRNAs) (20 pmol/well; catalog No. 1027280, Allstars negative control, Qiagen, Mississauga, *ON*, Canada), Epac1 (catalog No. sc-41700), Epac2 (sc-41702) or Rap1 (sc-36384) siRNAs (20 pmol/well; Santa Cruz Biotechnology, Santa Cruz, CA, USA) using the Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After treatment for 24 h with the siRNA, the medium containing siRNA and transfection reagents was removed and cells were cultured for 24 h in fresh culture medium.

Immunohistochemistry

The placental tissues of the first trimester and third trimester were fixed in 4% paraformaldehyde before being processed and embedded in paraffin following standard procedures (Tamura *et al.*, 2003). Sections of paraffin-embedded second-trimester placenta (22 weeks of gestation; $n = 1$) were purchased from Super Bio Chips Laboratories (#SF88122A, Seoul, South Korea). Paraffin sections were rehydrated, boiled for 20 min with 10 mM citrate buffer (pH 6.0), and were then blocked with 10% normal goat serum in phosphate-buffered saline for 2 h at room temperature. Sections were incubated with polyclonal anti-Epac1 (catalog No. ab21235, 1:100, Abcam) or anti-Epac2 (clone H-220, 1:100, Santa Cruz Biotechnology) antibodies overnight at 4°C and were incubated subsequently with amino acid polymer conjugated with anti-rabbit IgG Fab′ and horseradish peroxidase (Histofine Simple Stain MAX-PO MULTI, Nichirei, Tokyo, Japan) and developed with Histofine Simple Stain DAB solution (Nichirei). Negative controls were incubated with rabbit IgG instead of primary antibody. Sections were counterstained with methyl green.

Cell-fusion assay

A cell-fusion assay of BeWo cells was performed as described previously (Yoshie *et al.*, 2008). Briefly, BeWo cells grown on poly-L-lysine-coated glass cover slips were fixed and stained with anti-desmosomal protein antibody (clone ZK-31, 1:200, Sigma-Aldrich) together with an AlexaFluor 594 goat anti-mouse antibody (Molecular Probes, Carlsbad, CA, USA) to distinguish cell borders. The nuclei were counterstained with 4′,6-diamino-2-phenylindole 2HCl (DAPI). The number of multinuclear cells in the five microscopic areas that were selected randomly was counted. Data were expressed as the ratio of each control and the effects of selective cAMP analogs, and knocking down of Epac1, Epac2 or Rap1 on syncytialization were evaluated in four independent experiments.

Statistical analysis

Data were expressed as mean ± SEM. Significance was assessed using Turkey–Kramer multiple-comparisons testing. A *P*-value <0.05 was considered statistically significant.

Results

Localization of Epac1 and Epac2 in human placenta

Expression of Epac1 and Epac2 in placental tissue at different stages was examined by immunohistochemistry. In the first- and second-trimester placenta, Epac1 was expressed in villous CTB, STB, stroma and extravillous trophoblast (EVT) (Fig. 1A and C). In addition to the positive staining of Epac1 in various trophoblasts, blood vessels and stroma in chorionic villi were also stained for this protein (Fig. 1C). In the third trimester of gestation, a low level of staining for Epac1 was detected in STB, blood vessels and EVT (Fig. 1E). Intense staining of Epac2 was detected in villous CTB, stroma and EVT in first- and second-trimester placentas compared with its expression in STB (Fig. 1B and D). Furthermore, Epac2 was expressed in blood vessels (mainly in endothelium) (Fig. 1D). In the third-trimester placenta, Epac2 was expressed in STB, blood vessels, stroma and EVT (Fig. 1F). Non-specific immunostaining was not detected in negative control (Fig. 1G).

Effects of Epac-selective cAMP analog on the production of hCG and progesterone in BeWo cells

Differentiated STB secretes hCG and progesterone. It is well known that the enhancement of intracellular cAMP levels stimulates the synthesis and secretion of hCG and progesterone (Feinman *et al.*, 1986). We examined the effect of the Epac-selective cAMP analog (CPT) and of the PKA-selective cAMP analog (Phe) on hCG and progesterone production in BeWo cells, a well-characterized trophoblast-derived human choriocarcinoma cell line, which retains the ability to undergo differentiation and syncytialization. Treatment with Phe caused enhancement of *hCGα* and *hCGβ* mRNA expression compared with control ($P < 0.01$), as assessed using quantitative RT–PCR analysis (Fig. 2A and B). CPT increased *hCGα* and *hCGβ* mRNA expression moderately ($P < 0.05$, Fig. 2A and B). However, when compared with treatment with Phe alone, cotreatment with CPT and Phe did not affect the expression of *hCGα* and the expression of *hCGβ* was attenuated (Fig. 2A and B). Changes in hCG protein levels were basically similar to the results obtained for the *hCGα* and *hCGβ* mRNA levels, when hCG levels in cultured medium were analyzed using immunoblotting (Fig. 2C). We next examined the effect of CPT and Phe on the production of progesterone in BeWo cells. The culture media used in Fig. 2C were subjected to RIA to determine progesterone levels (Fig. 3A). Compared with control, the content of progesterone was increased by stimulation with CPT ($P < 0.05$) or Phe ($P < 0.01$). Simultaneous treatment with Phe and CPT enhanced the production of progesterone further when compared with the Phe treatment alone ($P < 0.05$). The mRNA level for *P450scc*, which is a key enzyme for progesterone synthesis that converts cholesterol to pregnenolone, was up-regulated after treatment with Phe ($P < 0.01$, Fig. 3B). Treatment of CPT caused a 2.1-fold, but not significant, increase in the *P450scc* mRNA level. However, the level of *P450scc* mRNA after cotreatment with CPT and Phe was similar to that observed for Phe treatment alone at the same time point (Fig. 3B). We confirmed that the CPT treatment used in this study did not

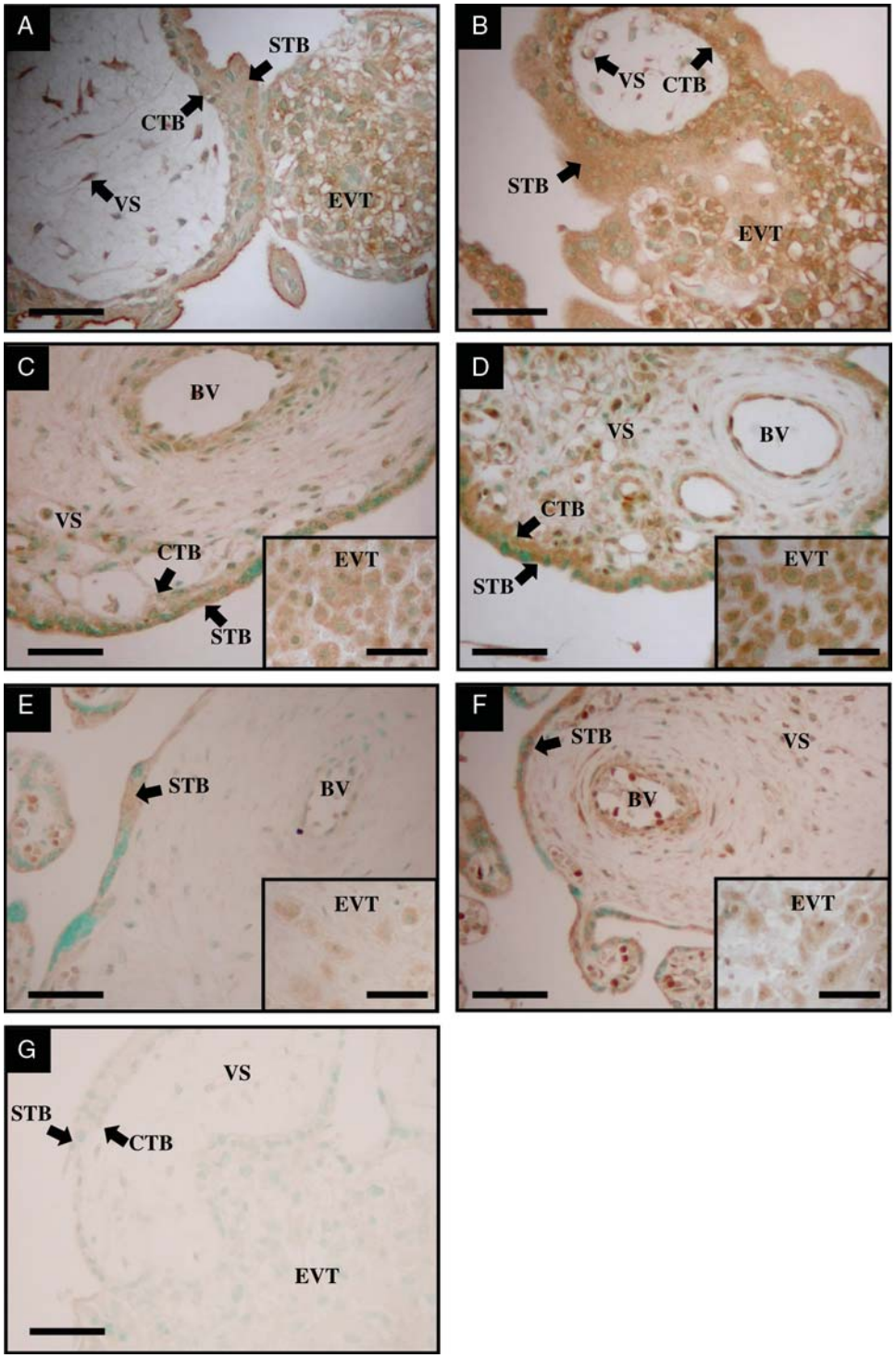

Figure 1 Expression of the Epac1 and Epac2 in human placenta. Placental tissues at different stages were immunostained with an anti-Epac1 (**A**, **C** and **E**; brown) or Epac2 (**B**, **D** and **F**; brown) antibody. (A, B and G) First trimester; (C and D) Second trimester; (E and F) Third trimester. Nuclei were counterstained with methyl green. (**G**) Negative control incubated with rabbit immunoglobulin G instead of primary antibody. The squared area in each picture is the magnification of EVT. CTB, cytotrophoblast; STB, syncytiotrophoblast; EVT, extravillous trophoblast; VS, villous stroma; BV, blood vessel. Scale bars = 50 μm.

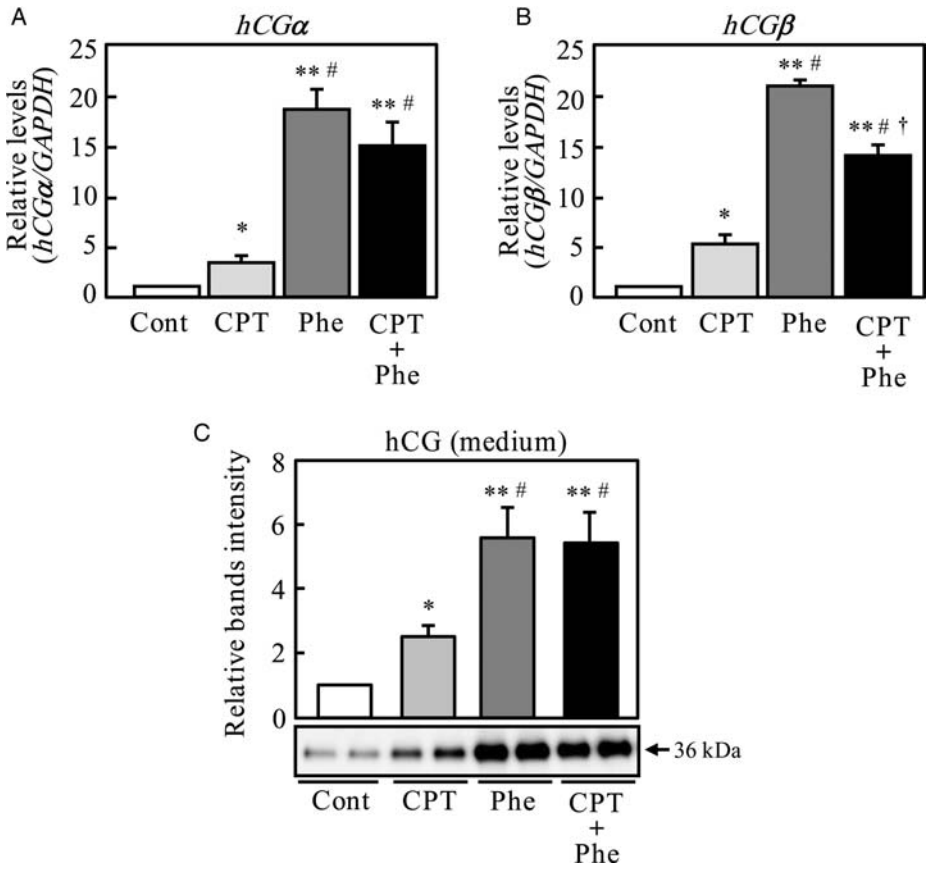

Figure 2 Effects of Epac- and PKA-selective cAMP analogs on the expression and secretion of hCG in BeWo cells**.** BeWo cells were cultured without (Cont) or with 0.5 mM of the Epac-selective cAMP analog (CPT) or 0.5 mM of the PKA-selective cAMP analog (Phe) for 48 h. (**A** and **B**) Total RNA was subjected to quantitative RT–PCR analysis to determine *hCGα* and *hCGβ* mRNA levels. Glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* was used as an internal control. (**C**) Culture media were subjected to immunoblot analysis using an anti-hCGβ antibody (lower panel) and the density of the band was calculated and normalized to the amount of total cell protein (upper panel). Each experiment was repeated at least three times and representative data are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Cont, $^{\#}P < 0.01$ versus CPT, $^{\dagger}P < 0.05$ versus Phe. Data are presented as ratios of the control group and are mean ± SEM.

affect phosphorylation of the cAMP response-element-binding protein, which is a classical downstream target of PKA signaling (data not shown).

Effects of Epac-selective cAMP analog on the syncytialization of BeWo cells

Mononuclear villous CTB continuously fuses and differentiates to form the multinuclear STB layer. Enhancement of intracellular cAMP levels induces their syncytialization in primary isolated trophoblasts (Keryer *et al.*, 1998) and BeWo cells (Ogura *et al.*, 2000). To examine whether Epac-mediated cAMP signaling is involved in syncytialization, BeWo cells were treated with CPT, Phe or a stable cAMP analog (Db) for 48 h. The number of multinuclear fused cells was assessed by DAPI nuclear staining and immunostaining of desmosomal protein to distinguish cell borders (Fig. 4A). In agreement with the previous report mentioned above, Db increased the ratio of syncytialization of BeWo cells (Fig. 4A and B). In particular, CPT promoted cell fusion significantly ($P < 0.01$), whereas Phe had little effect on syncytialization (Fig. 4A and B). To evaluate the possible relevance of Epac on syncytialization, we examined the effect of CPT- or Db-induced cell fusion in the presence or absence of the PKA inhibitor H89. BeWo cells were pretreated with H89 and were then incubated with CPT or Db for 48 h (Fig. 4C). Vehicle-treated cells served as a control group. CPT or Db treatment elevated syncytialization significantly versus control, even in the presence of the PKA inhibitor, while the ratio of syncytialized cells with Phe was comparable to that observed in the vehicle-treated group (Fig. 4C).

Effects of Epac and Rap1 knockdown on the cAMP-induced syncytialization of BeWo cells

To explore the role of Epac-mediated cAMP signaling on the syncytialization of BeWo cells, we examined the effects of siRNA-mediated knockdown of Epac1, Epac2 or their putative downstream factor, Rap1, on Db-, CPT- or an activator of adenylate cyclase, FSK-induced syncytialization (Fig. 5). Immunoblot analyzes showed that transfection of Epac1, Epac2 or Rap1 specific siRNA repressed expression of each protein by ~60, 80 and 90%, respectively (Fig. 5A). Control siRNA-transfected cells were syncytialized after treatment with Db, CPT or

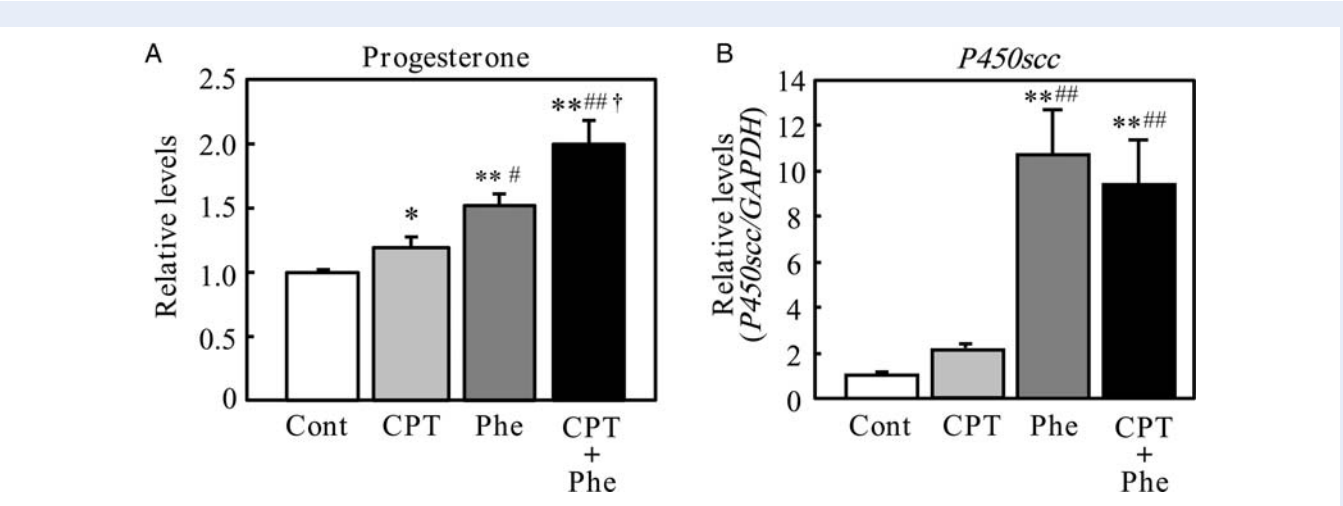

Figure 3 Effects of Epac- and PKA-selective cAMP analogs on progesterone production and P450scc mRNA expression in BeWo cells. BeWo cells were cultured without (Cont) or with 0.5 mM of CPT, Phe or a combination of CPT and Phe for 48 h. (**A**) Progesterone levels in the culture medium were measured using RIA and normalized to the amount of total cell protein. The data from three independent experiments were presented as ratios of the control levels and shown as mean ± SEM. (**B**) Total RNA was subjected to quantitative RT–PCR analysis of P450 side-chain cleavage enzyme *(P450scc) mRNA* level. Representative data from three independent experiments are shown.*$P < 0.05$, **$P < 0.01$ versus Cont, $^{\#}P < 0.05$, $^{\#\#}P < 0.05$ versus CPT, $^{\dagger}P < 0.05$ versus Phe.

FSK. However, knocking down of Epac1 or Rap1 resulted in a significant reduction of Db-, CPT- or FSK-induced syncytialization (Fig. 5B and C). In contrast, knockdown of Epac2 did not affect Db-, CPT- or FSK-stimulated syncytialization (Fig. 5B and C).

Discussion

The present study demonstrated for the first time that Epac1 and Epac2 were expressed in placental trophoblasts in chorionic villi and that Epac had possible roles in cAMP-mediated functional differentiation and syncytialization. The expression of the Epac1 mRNA in human placenta was confirmed previously using northern blotting (Kawasaki *et al.*, 1998); however, the localization of Epac1 and Epac2 in this tissue has not been reported to date. In first- and second-trimester placenta, both Epac1 and Epac2 were expressed in villous STB, CTB, stroma, blood vessels and EVT. In term placenta, Epac1 and Epac2 were mainly distributed in the STB layer, EVT and blood vessels. Interestingly, Epac1 and Epac2 were localized at the plasma membrane and in the cytoplasm of BeWo cells, respectively (data not shown). Epac1 is translocated rapidly and reversibly to the plasma membrane and activates Rap in response to a cAMP stimulus (Ponsioen *et al.*, 2009). We did not gather definitive information on the significance of intracellular localizations of Epac1 and Epac2 in this study; therefore, further experiments are required to clarify this issue.

The PKA-mediated cAMP signaling pathway is required for hCG synthesis (Chou, 1980; Nulsen *et al.*, 1998; Lambot *et al.*, 2005). Accordingly, the significant increase of hCG production was accompanied by the up-regulation of both *hCGα* and *hCGβ* mRNAs after treatment with a PKA-selective cAMP analog (Phe). These results suggest that PKA was the dominant mediator of cAMP-induced hCG synthesis. Our observation of moderate stimulation of the Epac-mediated hCG production suggests that Epac-mediated signaling may also be associated with hCG production. The effect of CPT on hCG production was also observed in another choriocarcinoma cell line, JEG-3 (data not shown). However, CPT did not up-regulate hCG synthesis additively in the presence of Phe; rather, it decreased the levels of the *hCGβ* mRNA. The Epac pathway might hamper the Phe-mediated hCG production, as the PKA-mediated pathway has been demonstrated to cross-talk with the Epac Pathway (Harper *et al.*, 2008). In addition, Phe increased the level of *P450scc* mRNA and the production of progesterone significantly. The expression of P450scc is up-regulated by cAMP analogs in primary isolated trophoblast and choriocarcinoma cell lines, which include BeWo cells and JEG-3 cells (Ringler *et al.*, 1989; Martínez *et al.*, 1997). Our results support the notion that PKA is the main mediator of the cAMP-induced P450scc expression. The stimulatory effect of CPT on progesterone secretion was also observed in JEG-3 cells (data not shown). In support of the present study, Chin and Abayasekara (2004) demonstrated that an Epac-selective cAMP analog increases progesterone secretion in human luteinized granulosa cells in a dose-dependent manner. Thus, Epac may be involved in the cAMP-dependent/PKA-independent pathway of progesterone production in mature follicles of human ovaries. Additionally, an Epac-selective cAMP analog and its metabolites increase the expression of *P450scc (CYP11a1)* mRNA and cortisol synthesis in bovine adrenocortical cells (Enyeart and Enyeart, 2009). Despite the enhanced increase in progesterone synthesis after simultaneous treatment with CPT and Phe, the *P450scc* mRNA levels did not change compared with Phe treatment alone. This indicates the possibility that Epac may mediate another pathway that is independent from *P450scc* expression in BeWo cells. Collectively, our data suggest that the cAMP/PKA signaling pathway plays a major role in functional trophoblast differentiation (which includes hCG and progesterone synthesis), whereas the cAMP/Epac signaling pathway is involved cooperatively in the differentiation of human trophoblasts (Fig. 6).

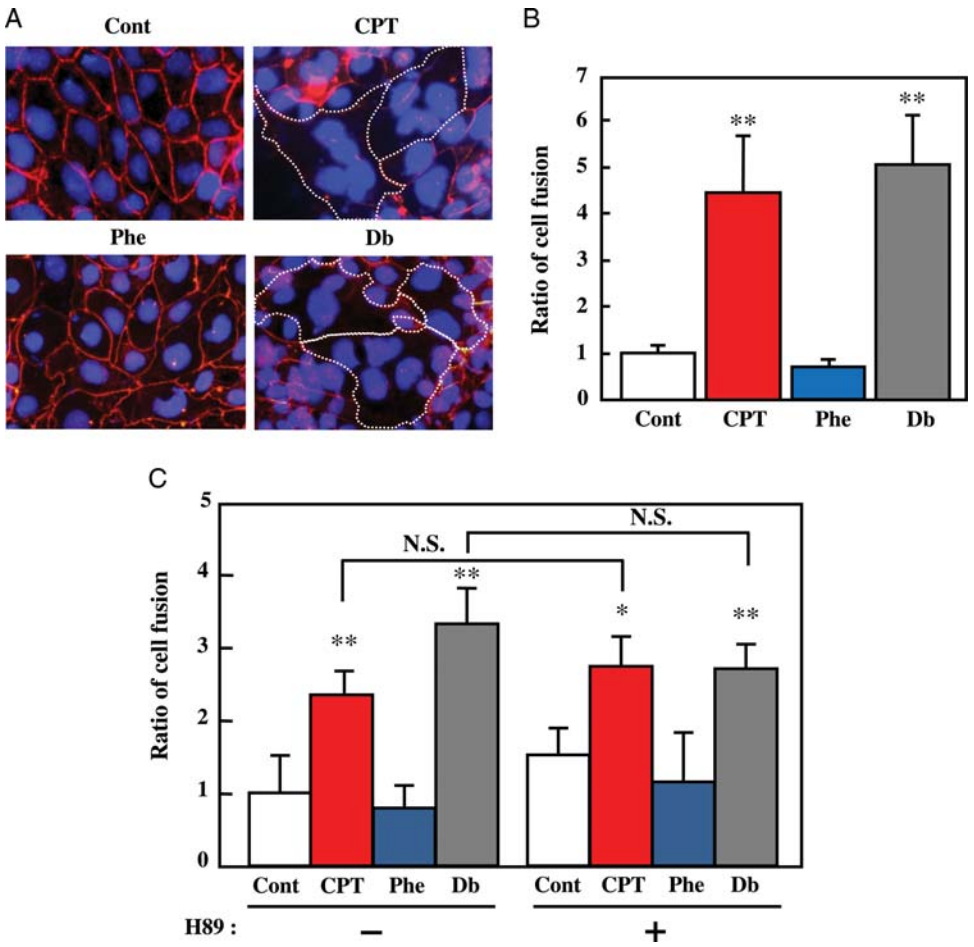

Figure 4 Effects of cAMP analogs and PKA inhibitor on the syncytialization of BeWo cells. BeWo cells were cultured without (Cont) or with 0.5 mM of CPT, Phe or Db for 48 h. Cells were immunostained with anti-desmosomal protein antibody (red) and 4′,6-diamidino-2-phenylindole (DAPI, blue) to visualize syncytialization. (**A**) Representative pictures are shown and multinuclear syncytialized cells are marked with a stippled line. (**B**) The number of syncytialized cells in five areas selected randomly was counted in each experiment. The data are presented as ratios of the control group and shown as mean ± SEM from four independent experiments. $^{**}P < 0.01$ versus Cont. (**C**) BeWo cells were pretreated with the PKA inhibitor H89 (10 μM) or vehicle (0.1% dimethylsulphoxide) for 1 h and were then cultured with each cAMP analog for 48 h. The number of syncytialized cells was counted using the same procedure applied in Figs. 4A and B. The data are presented as ratios of the control group and are shown as mean ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Cont. N.S., not significant.

We found that CPT mimicked the effect of the non-selective cAMP analog (Db)-induced BeWo cell fusion. In addition, the CPT- or Db-evoked syncytial fusion was retained in the presence of the PKA inhibitor. These findings suggest that the PKA-independent cAMP-Epac signaling pathway is involved in syncytialization. Furthermore, CPT- or Db-stimulated syncytialization was inhibited by the knocking down of Epac1 or Rap1, but not of Epac2. Interestingly, the PKA-selective cAMP analog (Phe) did not stimulate BeWo cell syncytialization. These results support the conclusion that the Epac1-mediated Rap1 activation is related to the cAMP-induced syncytialization of BeWo cells (Fig. 6). It has been suggested that cAMP-mediated syncytialization is PKA dependent (Keryer *et al.*, 1998) and probably involves up-regulation of the transcription factor glial cells missing a (GCMa), which results in up-regulation of the human endogenous retrovirus (HERV)-encoded protein syncytin1 in BeWo cells (Yu *et al.*, 2002; Chang *et al.*, 2005; Knerr *et al.*, 2005). Furthermore, the envelope protein of another HERV member, syncytin 2, which is expressed specifically in placental CTB, plays a crucial role in the process of FSK-induced trophoblast cell fusion (Malassiné *et al.*, 2007; Vargas *et al.*, 2009). The synergistic action of PKA and Epac in cAMP signaling is required to differentiate mouse 3T3-L1 fibroblasts to adipocytes (Petersen *et al.*, 2008). In contrast, persistent stimulation of PKA inhibits differentiation to adipocytes (Li *et al.*, 2008). These results may indicate that the strict regulation of PKA and Epac in cAMP-mediated signaling is required for appropriate cellular responses. In the present study, we did not gather data pertaining to how Epac/Rap1-medited cAMP signaling may stimulate BeWo cell syncytialization. Several reports have demonstrated that the formation of gap junctions, which enable cell-to-cell interactions, is required for trophoblast fusion (Cronier *et al.*, 2003; Frendo *et al.*, 2003). Connexin 43

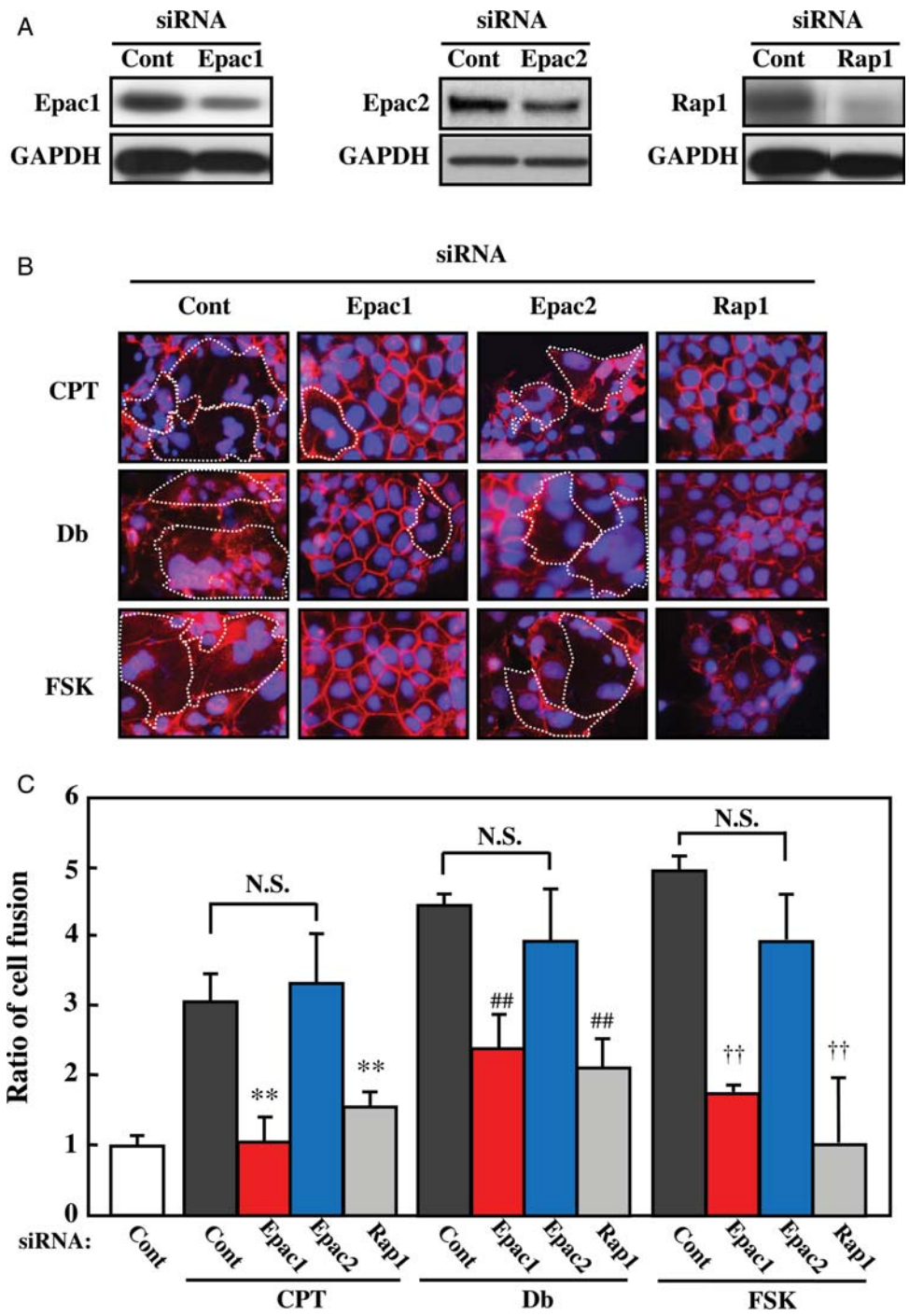

Figure 5 Effects of the knockdown of Epac1, Epac2 and Rap1 on the CPT-, Db- or FSK-induced syncytialization of BeWo cells. BeWo cells were transfected with non-targeting control (Cont), Epac1, Epac2 or Rap1 siRNAs and were then cultured with 0.5 mM CPT, 0.5 mM Db or 10 μM FSK for 48 h. (**A**) The expression of Epac1, Epac2 or Rap1 was determined using immunoblotting. The same blot was stripped and re-probed with anti-GAPDH antibody as a loading control. (**B**) Cells were immunostained with anti-desmosomal protein antibody (red) and DAPI (blue) to visualize syncytialization. Representative pictures are shown and syncytialized cells are marked with a stippled line. (**C**) The number of syncytialized cells in five areas selected randomly was counted. The data are presented as ratios of the control group and are shown as mean ± SEM from four independent experiments. $^{**}P < 0.01$ versus Cont siRNA/CPT, $^{\#\#}P < 0.01$ versus Cont siRNA/Db, $^{\dagger\dagger}P < 0.01$ versus Cont siRNA/FSK.

(Cx43), which is a component of gap junctions, is up-regulated during syncytialization, and the antisense-mediated knockdown of Cx43 expression inhibits syncytialization in isolated villous CTB (Frendo *et al.*, 2003). An Epac-selective cAMP analog stimulates gap-junction formation via the accumulation of Cx43 at cell–cell contact sites in rat cardiac myocytes (Somekawa *et al.*, 2005), although the

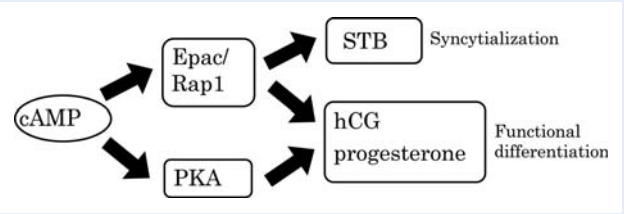

Figure 6 Diagram illustrating the potential role of Epac on syncytialization and functional differentiation of human trophoblasts.

physiological relationship between Cx43 and Epac in trophoblasts has not been examined. Furthermore, activation of Rap1 is necessary for the accumulation of Cx43 and of the cell-adhesion factor N-cadherin at cell–cell contact sites (Kooistra *et al*., 2007). Epac-mediated cAMP signaling may control the process of cell–cell contact via the formation of gap junctions, which are a prerequisite for syncytialization. The precise mechanisms of Epac1/Rap1-mediated fusion of trophoblasts warrant further investigation.

Further research focusing on the role of Epac in EVT migration and invasion may also be an important subject, as Epac may be associated with various types of cell migration, e.g. in melanoma (Baljinnyam *et al*., 2009), vascular smooth muscle cells (Yokoyama *et al*., 2008) and epithelial cells (Lyle *et al*., 2008).

In conclusion, the present study demonstrated that cAMP-mediated Epac signaling may regulate syncytialization via Rap1 and in part be involved in functional differentiation, which includes hCG and progesterone production, of human trophoblasts. This may imply that trophoblast differentiation, i.e. endocrine changes and syncytialization, are regulated differentially by cAMP signaling, although these functional and morphological aspects of differentiation occur synchronously in the process of placental formation.

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