EPAC2-mediated calreticulin regulates LIF and COX2 expression in human endometrial glandular cells

Kazuya Kusama^{1,2,*}, Mikihiro Yoshie^{1,*}, Kazuhiro Tamura¹, Kazuhiko Imakawa² and Eiichi Tachikawa¹

¹Department of Endocrine and Neural Pharmacology, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

²Laboratory of Theriogenology and Animal Breeding, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

*K Kusama and M Yoshie contributed equally to this work

Correspondence
should be addressed
to K Tamura
Email
hiro@toyaku.ac.jp

Abstract

The proper production of the implantation-related factors, leukemia inhibitory factor (LIF), cyclooxygenase 2 (COX2, PTGS2), and prostaglandin E2 (PGE2) in the uterine glands is essential for embryo implantation and the establishment of endometrial receptivity. It has been shown that cAMP-mediated protein kinase A (PKA) signaling regulates the production of these factors. We have previously reported that exchange protein directly activated by cAMP 2 (EPAC2, RAPGEF4), another cAMP mediator, is involved in the differentiation of endometrial stromal cells through the regulation of the expression of calreticulin (CALR). To address whether EPAC2–CALR signaling is involved in the expression of implantation-related factors, we examined the effect of EPAC2 and CALR knockdown on their expression in cultured human endometrial glandular epithelial EM1 cells, treated with forskolin, an adenylyl cyclase activator, an EPAC-selective cAMP analog (8-(4-chlorophenylthio)-2'-O-methyl cAMP (CPT)), or a PKA-selective cAMP analog (*N*⁶-phenyl-cAMP (Phe)). In addition, the status of cell senescence was examined. EPAC2 knockdown suppressed the expression of CALR protein and mRNA in EM1 cells. Forskolin- or Phe-, but not CPT-, induced expression of LIF or PTGS2 and secretion of PGE2 was inhibited in EPAC2- or CALR-silenced EM1 cells. In addition, knockdown of EPAC2 or CALR increased senescence-associated beta galactosidase activity and expression of p21 but decreased expression of p53. These findings indicate that expression of CALR regulated by EPAC2 in endometrial glandular epithelial cells is critical for the expression of LIF and PTGS2-mediated production of PGE2 through cAMP signaling. Furthermore, EPAC2 and CALR could play a role in the maintenance of gland function.

Key Words

- exchange protein directly activated by cAMP (EPAC)
- calreticulin (CALR)
- leukemia inhibitory factor (LIF)
- cyclooxygenase 2 (COX2, PTGS2)
- prostaglandin E2 (PGE2)
- endometrial glandular epithelial cell

Journal of Molecular Endocrinology
(2015) 54, 17–24

Introduction

Sex steroid hormones regulate the structure and function of the human uterine endometrium throughout the menstrual cycle (Lim & Wang 2010). This uterine change, including structural and secretory transformations, is a prerequisite for the establishment of uterine receptivity,

peri-implantation embryo survival, and embryo implantation as well as placentation (Cha *et al.* 2012). Endometrial glandular epithelial cells synthesize and secrete implantation-related factors, including leukemia inhibitory factor (LIF), cyclooxygenase 2 (COX2, PTGS2),

and prostaglandin E2 (PGE2), which are essential for embryo development and endometrial stromal cell differentiation (Stewart *et al.* 1992, Lim *et al.* 1999). LIF expression and PTGS2-mediated PGE2 production are elevated in endometrial glandular cells during the implantation window (Senturk & Arici 1998, Marions & Danielsson 1999, Milne *et al.* 2001). It has been demonstrated that *Lif*-null mice are infertile because of defective attachment of blastocysts to the uterine luminal epithelium (Chen *et al.* 2000), while *Ptgs2*-null mice show a failure in the decidualization of stromal cells (Matsumoto *et al.* 2002). In addition, activation of cAMP-mediated protein kinase A (PKA) signaling increases the expression of LIF and PTGS2 in human endometrial glandular cells (Zhou *et al.* 1999, Kutsukake *et al.* 2010). The proper expression of these factors is necessary for the successful establishment of pregnancy.

Exchange protein directly activated by cAMP (EPAC) is a cAMP-mediated signaling effector whose function is distinct from the classical PKA-signaling pathway (Kawasaki *et al.* 1998, de Rooij *et al.* 1998). EPAC has two isoforms, EPAC1 (RAPGEF3) and EPAC2 (RAPGEF4), which are implicated in several cellular responses, including cell proliferation, cell differentiation, hormone secretion/exocytosis, and formation of cell–cell junctions (Ahn *et al.* 2006, Bos 2006, Kooistra *et al.* 2007). In addition, EPAC1 is associated with the cAMP-dependent differentiation of cytotrophoblasts into syncytiotrophoblasts during placentation (Yoshie *et al.* 2010). We recently demonstrated that both EPAC1 and EPAC2 are expressed at the endometrial stroma, and glandular and luminal epithelium throughout the menstrual cycle, and activation of EPAC signaling enhanced decidualization of human endometrial stromal cells induced by ovarian steroids or cAMP analogs (Kusama *et al.* 2013). Furthermore, EPAC2-mediated expression of calreticulin (CALR), a novel downstream factor of EPAC2, is essential for decidualization *in vitro* (Kusama *et al.* 2014a). CALR is localized in not only stromal but also in glandular and luminal epithelial cells, in the human endometrium (Parmar *et al.* 2009a). The mechanism of expression of implantation-related factors, LIF and PTGS2, in endometrial epithelial cells has so far been studied through the downstream events of cAMP-mediated PKA signaling (Zhou *et al.* 1999, Kutsukake *et al.* 2010). However, proteins besides those involved in cAMP-mediated PKA signaling and expression of implantation-related factors have not been studied. In this study, we explored the role of EPAC2 and CALR in the expression of implantation-related factors in human endometrial glandular epithelial cells.

Materials and methods

Reagents and antibodies

8-(4-Chlorophenylthio)-2'-O-methyl cAMP (CPT) and N⁶-phenyl-cAMP (Phe) were purchased from the Biolog Life Science Institute (Bremen, Germany). An adenylyl cyclase stimulator, forskolin, and a selective PTGS2 inhibitor, NS-398, were obtained from Applichem (Darmstadt, Germany) and Cayman Chemical Company (Ann Arbor, MI, USA) respectively. Antibodies against CALR, p53 (clone 1C12), p21 (clone DCS60), and EPAC2 (clone 5B1) were purchased from Cell Signaling Technology (Beverly, MA, USA). An antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, clone GAPDH-71.1) was purchased from Sigma–Aldrich.

Cell culture

To study a possible role of EPAC2 or CALR in the production of implantation-related factors, an immortalized human endometrial glandular epithelial cell line (EM-E6/E7/TERT-1 cells; EM1) was used. Cells were grown at 37 °C in DMEM and Ham's F-12 supplemented with 10% (w/v) charcoal-stripped fetal bovine serum, 50 µg/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml neomycin, and 0.5 µg/ml amphotericin B. EM1 cells were treated with 15 µM forskolin, 200 µM CPT, and/or 200 µM Phe for 48 h.

Treatment of EM1 cells with siRNA or PTGS2 inhibitor

EM1 cells grown to 50% confluency in 24-well plates were transfected with non-targeting control siRNA (30 pmol/well, Qiagen), *EPAC1* siRNA (30 pmol/well; 5'-AUUGAGAUUCUUCUGCUCCUUGAGG-3' and 5'-CCU-CAAGGAGCAGAAGAAUCUCAAU-3', Invitrogen), *EPAC2* siRNA (30 pmol/well; 5'-UGUUCUUUAAGUCUGACUGAUUUCG-3' and 5'-CGAAUACAGUCAGACUAAAA-GAACA-3', Invitrogen), *RAP1* siRNA (30 pmol/well; sc-36384D, Santa Cruz Biotechnology), or *CALR* siRNA (30 pmol/well; 5'-GCAGACAAGCCAGGAUGCACGCU-UU-3' and 5'-AAAGCGUGCAUCCUGGCUUGUCUGC-3', Invitrogen) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. After treatment with siRNA for 24 h, the EM1 cells were washed with PBS and then cultured in fresh medium for 24 h, followed by medium containing or not containing forskolin or cAMP analogs for 48 h. The expression of *LIF* and *PTGS2* mRNA peaked at between 48 and 72 h in the preliminary experiment.

The concentrations of siRNA sufficient to suppress the expression of each protein were predetermined as described previously (Kusama *et al.* 2014a) and the knock-down efficiency was $79.0 \pm 8.1\%$ for EPAC2 and $75.0 \pm 8.5\%$ for CALR at the protein level. In addition, the amounts of PTGS2 sufficient to inhibit the effect of PTGS2 were also determined. A dose of $10 \mu\text{M}$ NS-398 was used to treat EM1 cells, which were cultured for 49 h before RNA extraction.

Immunoblotting

Protein lysates from EM1 cells were prepared with RIPA buffer (Cell Signaling Technology) according to the manufacturer's instructions. Equal amounts of protein ($20 \mu\text{g}$) were separated by SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore, Billerica, MA, USA). These membranes were probed with primary antibodies against CALR (1:2000), p53 (1:1000), p21 (1:1000), or EPAC2 (1:2000). Immunoreactive bands were detected using ECL (PerkinElmer Life Science, Inc., Boston, MA, USA) after incubation with HRP-labeled goat anti-mouse or anti-rabbit IgG ($0.5 \mu\text{g}/\text{ml}$, Vector Laboratories, Burlingame, CA, USA). Membranes were then treated with stripping solution (25 mM glycine-HCl, pH 2.0, containing 1% (w/v) SDS) and re-probed with an antibody against GAPDH ($0.2 \mu\text{g}/\text{ml}$). The relative band intensity was assessed by densitometric analysis of digitalized autographic images using Scion Image Software (Scion Corp., Fredrick, MD, USA) and normalized to GAPDH (Kusama *et al.* 2014a).

RNA extraction and real-time RT-PCR

Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA (100 ng) was amplified by real-time RT-PCR using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad Laboratories). Sense (S) and antisense (AS) primers were 5'-GACCTCTGGCAGGTCAAGTC-3' (S) and 5'-TCAGCGTATGCCTCATCGT-3' (AS) for *CALR*, 5'-TGAAGTGCAGCCCATGAATGA-3' (S) and 5'-TTCCAGTGCAGAACCA CAG-3' (AS) for *LIF*, 5'-CTTCACGCATCAGTTTTTCAAG-3' (S) and 5'-TCACCGTAAATATGATTAAAGTCCAC-3' (AS) for *PTGS2*, and 5'-AGCCACATCGCTCAGACA-3' (S) and 5'-GCCCAATACGACCAAATCC-3' (AS) for *GAPDH*. The fold change in the expression of each gene was calculated using the $\Delta\Delta\text{Ct}$ method with *GAPDH* as an internal control (Kusama *et al.* 2014a).

PGE2 ELISA

EM1 cells were treated with control, *EPAC2*, or *CALR* siRNA for 24 h and then with forskolin, Phe, and/or CPT for 48 h. The culture medium was centrifuged at $10\,000 g$ for 10 min at 4°C , and the concentration of PGE2 in the supernatant was determined using a sandwich ELISA Kit (Prostaglandin E2 Express EIA Kit, Cayman Chemical Company) according to the manufacturer's instructions.

Senescence-associated beta galactosidase staining

EM1 cells, transfected with control, *EPAC2*, or *CALR* siRNA for 48 h, were fixed in 4% (w/v) paraformaldehyde for 10 min and then washed twice with PBS. Staining was performed overnight at 37°C in PBS (pH 6.0) containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mM MgCl_2 , and 1 mg/ml X-gal. The number of senescence-associated beta galactosidase (SA- β -Gal)-positive cells was determined in five randomly chosen

Figure 1

Downregulation of CALR expression in *EPAC2*-silenced EM1 cells. EM1 cells were treated for 24 h with non-targeting control (Cont) or *EPAC2* siRNA and cultured in fresh medium for an additional 24 h. (A) Cell lysates were subjected to immunoblot analysis with an anti-CALR or EPAC2 antibody. The same blot was stripped and re-probed with an anti-GAPDH antibody, which served as a loading control. The graph shows the relative levels of CALR normalized to levels of GAPDH from three independent experiments. (B) Total RNA was subjected to real-time RT-PCR analysis to determine the levels of *CALR* mRNA. *GAPDH* was used as an internal control. Results from three independent experiments are presented. * $P < 0.01$ versus Cont siRNA.

fields and expressed as a ratio compared with the control siRNA-transfected group (Kusama *et al.* 2014a).

Statistical analysis

Data are expressed as the mean $\pm$ S.E.M. Significance was assessed using the Tukey–Kramer multiple comparisons test. A *P* value <0.05 was considered statistically significant.

Results

Downregulation of CALR expression in EPAC2-silenced EM1 cells

We previously reported that EPAC2 modulated expression of CALR in human endometrial stromal cells (Kusama *et al.* 2014a). To determine whether knockdown of EPAC2 downregulated expression of CALR protein, the level of CALR in EPAC2-silenced EM1 cells was examined by immunoblot analysis. EPAC2 knockdown significantly suppressed CALR expression (Fig. 1A). In addition, results from real-time RT-PCR analysis indicated that knockdown of EPAC2 inhibited the expression of CALR mRNA (Fig. 1B).

Inhibition of expression of implantation-related factors in EPAC2- or CALR-silenced EM1 cells

It is reported that cAMP-mediated PKA activation increases the expression of *LIF* or *PTGS2* mRNA (Zhou *et al.* 1999, Kutsukake *et al.* 2010). We investigated whether EPAC2 and CALR were involved in cAMP signaling-induced expression of *LIF* or *PTGS2* and secretion of PGE2 in EM1 cells. Transfection of EM1 cells with CALR or EPAC2 siRNA suppressed expression of CALR or EPAC2 protein respectively (Fig. 2A). The effect of forskolin, an adenylyl cyclase activator, on expression of *LIF* or *PTGS2* was reduced in CALR- or EPAC2-silenced EM1 cells (Fig. 2B and C). Forskolin-induced secretion of PGE2 was also blocked by knockdown of CALR or EPAC2 (Fig. 2D). To confirm that these results were EPAC2- or CALR-specific, EM1 cells transfected with EPAC1 or RAP1 siRNA were similarly treated with forskolin. Forskolin-induced expression of *LIF* or *PTGS2* did not differ in EPAC1- or RAP1-silenced EM1 cells (Fig. 2E and F). These results indicated that EPAC2 and CALR, but not EPAC1 or RAP1, regulated the cAMP-stimulated expression of *LIF* and *PTGS2* and secretion of PGE2 in EM1 cells. PGE2 has

Figure 2

Inhibition of the expression of forskolin-stimulated implantation-related factors in EPAC2- or CALR-silenced EM1 cells. (A, B, C, D, E and F) EM1 cells were treated for 24 h with non-targeting control (Cont), EPAC2, CALR, EPAC1, or RAP1 siRNA and cultured for an additional 24 h. EM1 cells were then treated with forskolin (15 μ M) for 48 h. The expression of EPAC2 or CALR was determined by immunoblotting (A). The same blot was stripped and re-probed with an anti-GAPDH antibody, which served as a loading control. Total RNA was subjected to real-time RT-PCR analysis to determine the levels of *LIF* (B and E) and *PTGS2* (C and F) mRNA. *GAPDH* was used as an internal control. (D) The level of PGE2 in culture media was determined by

ELISA. (G) One hour before the forskolin stimulation, EM1 cells were treated with or without 10 μ M NS-398. EM1 cells were further cultured for 48 h and harvested for extraction of RNA, from which levels of *LIF* and *PTGS2* mRNA were analyzed. (B, C and D) Cont siRNA (white bars), *Epac2* siRNA (black bars), and *Calr* siRNA (gray bars), (E and F) Cont siRNA (white bars), *Epac1* siRNA (gray bars), and *Rap1* siRNA (black bars). (G) Control (white bars) and NS-398 (gray bars). The results from three independent experiments are presented. **P* <0.01 versus Cont siRNA and #*P* <0.01 versus Cont. Values represent the mean $\pm$ S.E.M.

been shown to activate the intracellular downstream pathways through cAMP in endometrial stromal cells (Cheng *et al.* 2007). We therefore examined whether treatment with NS-398, a PTGS2 inhibitor, affected the levels of *LIF* and *PTGS2* mRNA in EM1 cells treated with forskolin. However, the expression of *LIF* and *PTGS2* induced by forskolin was not affected by the presence of a PTGS2 inhibitor (Fig. 2G).

Induction of cAMP analog-stimulated implantation-related factors was inhibited in EPAC2- or CALR-silenced EM1 cells

Results of previous studies indicated that EPAC2 signaling potentiated the differentiation of endometrial stromal cells when these cells were stimulated by a PKA-selective agonist (Kusama *et al.* 2013, 2014a). To investigate the role of EPAC2- or CALR-mediated cAMP signaling in the cAMP-mediated induction of implantation-related factors, EM1 cells in which *EPAC2* or *CALR* was downregulated were treated with PKA-selective (Phe) and/or EPAC-selective (CPT) cAMP analogs. The expression of *LIF* or *PTGS2* was increased by Phe, but not by CPT (Fig. 3A and B). Moreover, *LIF* or *PTGS2* expression when cells were treated with Phe and CPT (Phe/CPT) was similar to that of the Phe treatment group. Thus, the EPAC agonist did not upregulate the expression of *LIF* and *PTGS2* when these cells were stimulated with a PKA agonist. In *CALR*- or *EPAC2*-silenced EM1 cells, Phe- or Phe/CPT-induced expression of *LIF* or *PTGS2* was significantly downregulated. Similar results were observed for the secretion of PGE2 (Fig. 3C). These results indicated that, regardless of EPAC signaling, EPAC2 and/or CALR regulate the expression of *LIF* and production of PGE2 through the expression of *PTGS2* in EM1 cells.

Induction of senescence in EPAC2- or CALR-silenced EM1 cells

It has been reported that cell senescence is induced in *Calr*-deficient mouse fibroblasts (Iakova *et al.* 2004), and that knockdown of *EPAC2* or *CALR* promotes cell senescence in endometrial stromal cells (Kusama *et al.* 2014a). To investigate whether EM1 cells in which *EPAC2* or *CALR* had been downregulated exhibited cell senescence, we examined the activity of SA- β -Gal, and the expression of p21 and p53, well-established markers of cellular senescence (Kusama *et al.* 2014a). The intensity of SA- β -Gal staining significantly increased in the *EPAC2* or *CALR* siRNA-treated EM1 cells compared with the

Figure 3

Inhibition of cAMP analog-stimulated implantation-related factors in *EPAC2*- or *CALR*-silenced EM1 cells. EM1 cells were treated for 24 h with non-targeting control (Cont), *EPAC2* or *CALR* siRNA and cultured for an additional 24 h. Cells were then treated with Phe (200 μ M) and/or CPT (200 μ M) for 48 h, and total RNA was subjected to real-time RT-PCR analysis to determine the levels of *LIF* (A) and *PTGS2* (B) mRNA. *GAPDH* was used as an internal control. The level of PGE2 in the culture media was determined by ELISA (C). Cont siRNA (white bars), *EPAC2* (black bars), and *CALR* siRNA (gray bars). The results from three independent experiments are presented. * $P < 0.01$ versus Cont siRNA and [#] $P < 0.01$ versus Cont. Values represent the mean $\pm$ S.E.M.

control cells (Fig. 4A and B). Furthermore, knockdown of either *EPAC2* or *CALR* in EM1 cells suppressed the level of p53 but increased the level of p21 (Fig. 4C, D and E). These results were indicative of an association between EPAC2/CALR and senescence in EM1 cells.

Discussion

To elucidate the possible role of EPAC2 or CALR in the endometrial gland as it prepares for implantation, we investigated the expression of *LIF* and *PTGS2* and the secretion of PGE2 in EPAC2- or CALR-silenced endometrial epithelial EM1 cells. EM1 cells exhibit gland-like structure in Matrigel and respond to 17 β -estradiol by proliferation, both of which are characteristic of epithelial cells, not stromal cells (Kyo *et al.* 2003, Kutsukake *et al.* 2010). The results of this study indicated that knockdown of EPAC2 suppressed the expression of CALR mRNA and protein and that EPAC2-regulated CALR was essential for the production of *LIF* and *PTGS2* and secretion of PGE2 in EM1 cells. Treatment of EM1 cells with an EPAC-selective cAMP analog (CPT) did not affect the expression of *LIF* or *PTGS2* and secretion of PGE2; however, forskolin-induced of these implantation-related factors was inhibited in EPAC2- or CALR-silenced EM1 cells. These results indicated that although the activation of EPAC signaling itself had no effect, EPAC2 or CALR was required for sufficient

expression of *LIF* and *PTGS2* and production of PGE2 in endometrial glandular epithelial cells. It has been shown that PGE2 itself activates the intracellular downstream pathways through cAMP in endometrial stromal cells (Cheng *et al.* 2007). However, expression of *LIF* induced by forskolin was not altered in the presence of a PTGS2 inhibitor in EM1 cells, indicating that expression of *LIF* mediated through activation of PKA could occur independently from endogenous PGE2. It has also been reported that knockdown of EPAC2 or CALR inhibits PKA signaling in human and rat endometrial stromal cells (Kusama *et al.* 2013, 2014a,b). In this study, the expression of PKA-selective cAMP analog (Phe)-stimulated implantation-related factors was inhibited in EPAC2- or CALR-silenced EM1 cells. Thus, these results indicate that EPAC2 or CALR could have interacted with the PKA-signaling pathway; however, the precise mechanisms by which EPAC2 or CALR affected the PKA-mediated effects on the expression of implantation-related factors is yet to be determined.

As shown in Fig. 4C and E, knockdown of EPAC2 or CALR suppressed the expression of p53 in EM1 cells. It has

Figure 4

Induction of senescence in EPAC2- or CALR-silenced EM1 cells. EM1 cells were treated for 24 h with non-targeting control (Cont), EPAC2, or CALR siRNA and then cultured for 72 h. (A) EM1 cells were stained with SA-β-Gal. Scale bars = 100 μ m. (B) The graph shows the relative levels of SA-β-Gal-positive cells. The results from three independent experiments are presented as ratios and show the mean $\pm$ s.e.m. (C, D and E) Cell lysates were

subjected to immunoblot analysis with an anti-p21, p53, EPAC2, or CALR antibody. The same blot was stripped and re-probed with anti-GAPDH antibody, which served as a loading control (C). The graphs show the relative levels of p21 (D) or p53 (E) normalized to the level of GAPDH. The results (mean $\pm$ s.e.m.) from three independent experiments are presented as ratios. ** P < 0.01, * P < 0.05 versus Cont.

been reported that the expression of the *LIF* gene with the p53-binding site in its upstream region is reduced in p53-gene-ablated mice and that reduced expression of p53 leads to infertility via a decrease in the expression of *LIF* in mice and humans (Hu *et al.* 2007, Kang *et al.* 2009). It has also been demonstrated that expression of p53 is dependent on the level of expression of *CALR* in heterozygous and homozygous *CALR*-deficient embryos (Mesaali & Phillipson 2004) and that *CALR* is abundantly expressed in the implantation site of primate endometrial glands compared with the non-implantation site (Parmar *et al.* 2009b). Our findings imply that the reduction in the expression of *LIF* could have resulted in part from the decrease in the expression of p53 in *EPAC2*- or *CALR*-silenced EM1 cells.

The results of this study also indicated that the knockdown of *EPAC2* or *CALR* induced senescence in EM1 cells. It has been shown that uterine-specific p53-knockout mice exhibit senescence-associated growth restriction with an increase in endometrial SA- β -Gal activity and p21 expression and experience preterm labor (Hirota *et al.* 2010). On the basis of these findings, it is conceivable that cellular senescence in *EPAC2*- or *CALR*-silenced EM1 cells could impair the production of implantation-related factors due in part to the reduction in *CALR*-mediated expression of p53 or p21. It is possible that *EPAC2*-mediated expression of *CALR* could have prevented the senescence of endometrial glands, which in turn supports the interaction between the endometrium and embryo in humans. It is also conceivable that the prevention of cell senescence mediated through *CALR* signaling could induce functional changes in endometrial glands during the menstrual cycle in humans. Together, these observations support our postulate that endometrial glandular *EPAC2* and *CALR* could be the regulators of endometrial gland development, and possibly maintenance, during the menstrual cycle and, possibly, implantation.

It has been shown that the influx of calcium in uterine epithelial cells triggers adhesiveness of human trophoblast-like cells (Tinel *et al.* 2000) and that *EPAC2* or *CALR* regulates extracellular or intracellular influx of Ca^{2+} (Michalak *et al.* 2009, Zhang *et al.* 2009). Hence, it is possible that the downregulation of *EPAC2* or *CALR* in EM1 cells could have caused the reduction in the expression of *LIF* or *PTGS2* and secretion of PGE2 through the dysregulation of the intracellular influx of Ca^{2+} . In addition, S100A11, a Ca^{2+} -binding protein that regulates Ca^{2+} homeostasis, is expressed in human endometrial cells and involved in the expression of *LIF*

and implantation (Liu *et al.* 2012). Furthermore, the activation of the epithelial Na^{+} channel triggers an influx of Ca^{2+} that leads to the upregulation of the expression of *PTGS2* and the release of PGE2 via the activation of PKA in mouse endometrial epithelial cells (Ruan *et al.* 2012). These results indicate that in *EPAC2*- or *CALR*-silenced EM1 cells, downregulation of the production of PKA-mediated implantation-related factors could have resulted from the dysregulation of the Ca^{2+} influx.

In conclusion, we demonstrated that *EPAC2* regulated the expression of *CALR* in endometrial glandular epithelial cells and that the expression of *CALR* was crucial for the induction of the implantation-related factors, *LIF*, *PTGS2*, and PGE2 in the activation of cAMP signaling. Furthermore, *EPAC2* and *CALR* partially regulated the expression of p53 or p21 and maintained the glandular function for the prevention of cellular senescence.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by KAKENHI (Grant-in-Aid for Young Scientists (B), 25861511 to M Y) from the Japan Society for the Promotion of Science.

References

- Ahn YH, Han JH & Hong SH 2006 Rap1 and p38 MAPK mediate 8-chloro-cAMP-induced growth inhibition in mouse fibroblast DT cells. *Journal of Cellular Physiology* **209** 1039–1045. (doi:10.1002/jcp.20821)
- Bos JL 2006 EPAC proteins: multi-purpose cAMP targets. *Trends in Biochemical Sciences* **31** 680–686. (doi:10.1016/j.tibs.2006.10.002)
- Cha J, Sun X & Dey SK 2012 Mechanisms of implantation: strategies for successful pregnancy. *Nature Medicine* **18** 1754–1767. (doi:10.1038/nm.3012)
- Chen JR, Cheng JG, Shatzer T, Sewell L, Hernandez L & Stewart CL 2000 Leukemia inhibitory factor can substitute for nidatory estrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis. *Endocrinology* **141** 4365–4372. (doi:10.1210/endo.141.12.7855)
- Cheng Z, Sheldrick EL, Marshall E, Wathes DC, Abayasekara DR & Flint AP 2007 Control of cyclic AMP concentration in bovine endometrial stromal cells by arachidonic acid. *Reproduction* **133** 1017–1026. (doi:10.1530/REP-06-0220)
- Hirota Y, Daikoku T, Tranguch S, Xie H, Bradshaw HB & Dey SK 2010 Uterine-specific p53 deficiency confers premature uterine senescence and promotes preterm birth in mice. *Journal of Clinical Investigation* **120** 803–815. (doi:10.1172/JCI40051)
- Hu W, Feng Z, Teresky AK & Levine AJ 2007 p53 regulates maternal reproduction through LIF. *Nature* **29** 721–724. (doi:10.1038/nature05993)
- Iakova P, Wang GL, Timchenko L, Michalak M, Pereira-Smith OM, Smith JR & Timchenko NA 2004 Competition of CUGBP1 and calreticulin for

- the regulation of p21 translation determines cell fate. *EMBO Journal* **23** 406–417. (doi:10.1038/sj.emboj.7600052)
- Kang HJ, Feng Z, Sun Y, Atwal G, Murphy ME, Rebbeck TR, Rosenwaks Z, Levine AJ & Hu W 2009 Single-nucleotide polymorphisms in the p53 pathway regulate fertility in humans. *PNAS* **106** 9761–9766. (doi:10.1073/pnas.0904280106)
- Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE & Graybiel AM 1998 A family of cAMP-binding proteins that directly activate Rap1. *Science* **282** 2275–2279. (doi:10.1126/science.282.5397.2275)
- Kooistra MR, Dubé N & Bos JL 2007 Rap1: a key regulator in cell–cell junction formation. *Journal of Cell Science* **120** 17–22. (doi:10.1242/jcs.03306)
- Kusama K, Yoshie M, Tamura K, Kodaka Y, Hirata A, Sakurai T, Bai H, Imakawa K, Nishi H, Isaka K *et al.* 2013 Regulation of decidualization in human endometrial stromal cells through exchange protein directly activated by cyclic AMP Epac. *Placenta* **34** 212–221. (doi:10.1016/j.placenta.2012.12.017)
- Kusama K, Yoshie M, Tamura K, Nakayama T, Nishi H, Isaka K & Tachikawa E 2014a The role of exchange protein directly activated by cyclic AMP 2-mediated calreticulin expression in the decidualization of human endometrial stromal cells. *Endocrinology* **155** 240–248. (doi:10.1210/en.2013-1478)
- Kusama K, Yoshie M, Tamura K, Daikoku T, Takarada T & Tachikawa E 2014b Possible roles of the cAMP-mediators EPAC and RAP1 in decidualization of rat uterus. *Reproduction* **147** 897–906. (doi:10.1530/REP-13-0654)
- Kutsukake M, Tamura K, Yoshie M & Tachikawa E 2010 Knockdown of IGF-binding protein 7 inhibits transformation of the endometrial gland in an *in vitro* model. *Molecular Reproduction and Development* **77** 265–272. (doi:10.1002/mrd.21143)
- Kyo S, Nakamura M, Kiyono T, Maida Y, Kanaya T, Tanaka M, Yatabe N & Inoue M 2003 Successful immortalization of endometrial glandular cells with normal structural and functional characteristics. *American Journal of Pathology* **163** 2259–2269. (doi:10.1016/S0002-9440(10)63583-3)
- Lim HJ & Wang H 2010 Uterine disorders and pregnancy complications: insights from mouse models. *Journal of Clinical Investigation* **120** 1004–1015. (doi:10.1172/JCI41210)
- Lim H, Gupta RA, Ma WG, Paria BC, Moller DE, Morrow JD, DuBois RN, Trzaskos JM & Dey SK 1999 Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPAR δ . *Genes and Development* **13** 1561–1574. (doi:10.1101/gad.13.12.1561)
- Liu XM, Ding GL, Jiang Y, Pan HJ, Zhang D, Wang TT, Zhang RJ, Shu J, Sheng JZ & Huang HF 2012 Down-regulation of S100A11, a calcium-binding protein, in human endometrium may cause reproductive failure. *Journal of Clinical Endocrinology and Metabolism* **97** 3672–3683. (doi:10.1210/jc.2012-2075)
- Marions L & Danielsson KG 1999 Expression of cyclo-oxygenase in human endometrium during the implantation period. *Molecular Human Reproduction* **5** 961–965. (doi:10.1093/molehr/5.10.961)
- Matsumoto H, Ma WG, Daikoku T, Zhao X, Paria BC, Das SK, Trzaskos JM & Dey SK 2002 Cyclooxygenase-2 differentially directs uterine angiogenesis during implantation in mice. *Journal of Biological Chemistry* **277** 29260–29267. (doi:10.1074/jbc.M203996200)
- Mesaali N & Phillipson C 2004 Impaired p53 expression, function, and nuclear localization in calreticulin-deficient cells. *Molecular Biology of the Cell* **15** 1862–1870. (doi:10.1091/mbc.E03-04-0251)
- Michalak M, Groenendyk J, Szabo E, Gold LI & Opas M 2009 Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. *Biochemical Journal* **417** 651–666. (doi:10.1042/BJ20081847)
- Milne SA, Perchick GB, Boddy SC & Jabbour HN 2001 Expression, localization, and signaling of PGE $_2$ and EP2/EP4 receptors in human nonpregnant endometrium across the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism* **86** 4453–4459. (doi:10.1210/jcem.86.9.7856)
- Parmar T, Gadkar-Sable S, Savardekar L, Katkam R, Dharma S, Meherji P, Puri CP & Sachdeva G 2009a Protein profiling of human endometrial tissues in the midsecretory and proliferative phases of the menstrual cycle. *Fertility and Sterility* **92** 1091–1103. (doi:10.1016/j.fertnstert.2008.07.1734)
- Parmar T, Nimbkar-Joshi S, Katkam RR, Gadkar-Sable S, Chaudhari U, Manjramkar DD, Savardekar L, Jacob S, Puri CP & Sachdeva G 2009b Differential expression of calreticulin, a reticuloplasmin in primate endometrium. *Human Reproduction* **24** 2205–2216. (doi:10.1093/humrep/dep187)
- de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A & Bos JL 1998 EPAC is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* **396** 474–477. (doi:10.1038/24884)
- Ruan YC, Guo JH, Liu X, Zhang R, Tsang LL, Dong JD, Chen H, Yu MK, Jiang X, Zhang XH *et al.* 2012 Activation of the epithelial Na $^+$ channel triggers prostaglandin E $_2$ release and production required for embryo implantation. *Nature Medicine* **18** 1112–1117. (doi:10.1038/nm.2771)
- Senturk LM & Arici A 1998 Leukemia inhibitory factor in human reproduction. *American Journal of Reproductive Immunology* **39** 144–1451. (doi:10.1111/j.1600-0897.1998.tb00346.x)
- Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Köntgen F & Abbondanzo SJ 1992 Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359** 76–79. (doi:10.1038/359076a0)
- Tinel H, Denker HW & Thie M 2000 Calcium influx in human uterine epithelial RL95-2 cells triggers adhesiveness for trophoblast-like cells. Model studies on signalling events during embryo implantation. *Molecular Human Reproduction* **6** 1119–1130. (doi:10.1093/molehr/6.12.1119)
- Yoshie M, Kaneyama K, Kusama K, Higuma C, Nishi H, Isaka K & Tamura K 2010 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. *Human Reproduction* **25** 2229–2238. (doi:10.1093/humrep/deq190)
- Zhang CL, Katoh M, Shibasaki T, Minami K, Sunaga Y, Takahashi H, Yokoi N, Iwasaki M, Miki T & Seino S 2009 The cAMP sensor Epac2 is a direct target of antidiabetic sulfonylurea drugs. *Science* **325** 607–610. (doi:10.1126/science.1172256)
- Zhou XL, Lei ZM & Rao CV 1999 Treatment of human endometrial gland epithelial cells with chorionic gonadotropin/luteinizing hormone increases the expression of the cyclooxygenase-2 gene. *Journal of Clinical Endocrinology and Metabolism* **84** 3364–3377. (doi:10.1210/jcem.84.9.5943)

Received in final form 5 November 2014

Accepted 6 November 2014

Accepted Preprint published online 6 November 2014