—Mini Review—

# Molecular mechanisms of human endometrial decidualization activated by cyclic adenosine monophosphate signaling pathways

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***Abstract:*** During the menstrual cycle, the human endometrium undergoes dramatic changes, including cyclical proliferation, differentiation and menstruation, under the stringent control of ovarian steroid hormones. Endometrial stromal cells (ESCs) spontaneously differentiate into decidual cells in response to increased progesterone and intracellular cyclic adenosine monophosphate (cAMP) levels during the mid-secretory phase of the cycle. This process, termed decidualization, is strictly defined as the morphological and biochemical reprograming of the ESCs and is required for successful pregnancy. In pregnancy, the decidua functions as a critical barrier between the mother and fetus by modulating trophoblast invasion and the immune response. We recently demonstrated the involvement of a novel cAMP target, an exchange protein directly activated by cAMP (EPAC), in the process of decidualization. This review presents the molecular mechanisms of decidualization regulated by progesterone and the cAMP signaling pathway and highlights the role of EPAC in the process of decidualization.

***Key words:*** Decidualization, cAMP, PKA, EPAC, Progesterone

## Introduction

Under the strict control of ovarian steroid hormones, estradiol and progesterone (P4), the human endometrium dynamically and cyclically changes during the menstrual cycle (Fig. 1A). One of the distinctive changes required for embryo implantation is the differentiation of endometrial stromal cells (ESCs); a process known as 'decidualization' (Fig. 1B). Even under uterine circumstances where no fertilized embryo exists, human ESCs spontaneously transform and differentiate into decidual cells in a process that is controlled by P4 during the mid-secretory phase of the cycle. Decidualization is strictly defined as the morphological and biochemical reprogramming of stromal cells and is indispensable for embryo implantation. The process of decidualization is accompanied by a morphological transition from a fibroblastic to an enlarged and rounded morphology, as well as the secretion of specific marker antigens, including prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1) (Fig. 1B) [1–5]. Emerging evidence suggests that human ESCs become sensitive to embryonic signals upon decidualization and respond to a low-quality embryo by inhibiting the production of implantation-related factors [6]. Moreover, impaired decidualization disables embryo–maternal interactions and causes recurrent pregnancy loss [7]. In pregnancy, decidualization extends to the basal layer of the endometrium. The decidua regulates trophoblast invasion, placenta formation and the immune response against the fetus [8]. Thus, the decidua is necessary for the establishment and maintenance of successful pregnancy and functions as a biosensor during embryo selection at implantation, and is, therefore, a determinant of reproductive success.

Under normal physiological conditions, the process of decidualization is dependent on the action of P4. P4 is secreted from the newly formed corpus luteum following ovulation and induces the proliferation and decidualization of ESCs. These actions of P4 are mediated through binding to nuclear P4 receptors (PGR). A series of *in vivo*


Received: June 12, 2015
Accepted: July 21, 2015
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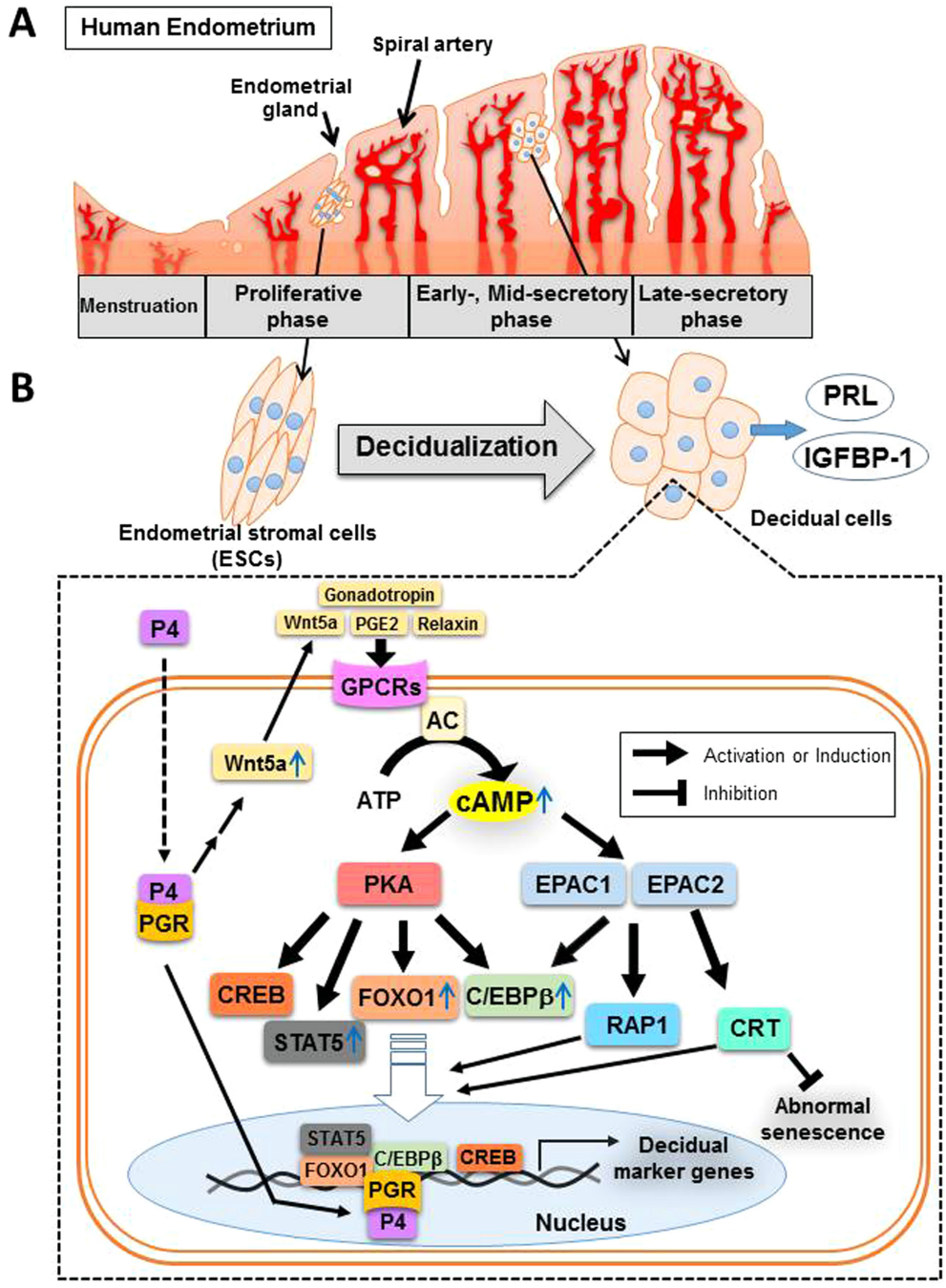


**Fig. 1.** Diagram illustrating the histological changes in the human endometrium during the menstrual cycle (A), and P4/P4 receptor (PGR) and cAMP signaling in decidualization (B). (A) Endometrial stromal cells (ESCs) spontaneously differentiate into decidual cells in response to increased P4 and intracellular cAMP levels during the mid-secretory phase of the cycle. (B) Crosstalk of the P4/PGR and PKA- and EPAC-mediated cAMP signaling involved in decidualization. The receptor P4/PGR complex binds to the promoter region of the decidual markers PRL and IGFBP-1. Various physiological factors including PGE2, relaxin, gonadotropin and Wnt5a activate their G protein-coupled receptors (GPCRs) linked to stimulatory G protein and then elevate intracellular cAMP. Activation of cAMP/PKA signaling induces or activates several transcriptional factors which interact with PGR. EPAC-mediated cAMP signaling cooperatively stimulates the cAMP/PKA pathway through RAP1 activation and/or calreticulin (CRT) expression. Furthermore, EPAC may enhance the expression and activity of C/EBP$\beta$ during decidualization.

and *in vitro* studies have demonstrated the critical role played by the P4/PGR signaling pathway in decidualization in mice and humans [9, 10].

The initiation of decidualization is accompanied by a sustained increase in the level of the intracellular second messenger, cyclic adenosine monophosphate (cAMP) [11]. Cell-permeable and stable cAMP analogs can induce decidualization more rapidly and efficiently than progesterone *in vitro*. In this context, physiological factors that trigger the elevation of intracellular cAMP by activating G protein-coupled receptors (GPCRs) linked to stimulatory G (Gs) proteins, such as prostaglandin E2 (PGE2) [12], relaxin [13], and gonadotropin [14], enhance decidualization (Fig. 1B). These results suggest the significance of the cAMP signaling pathway in the process of decidualization. Here we briefly focus on the molecular mechanisms of the decidualization process from the point of view of the P4/PGR and cAMP signaling pathways.

## P4/PGR Signaling in Decidualization

The actions of P4 in endometrial cells are thought to be mediated by its interaction with nuclear PGR, a member of the ligand-activated transcription factor superfamily. P4-mediated differentiation during decidualization is induced in the secretory phase of the menstrual cycle. The P4/PGR signaling pathway induces the transformation of undifferentiated ESCs to decidual cells. PGR has two isoforms, PGR-A and PGR-B, which are transcribed by different promoter usage in a single gene [15]. The PGR-A and -B receptor isoforms differ only in that PGR-B has an additional transactivation domain located at its amino terminus. PGR-B has been shown to function as a strong activator of transcription at several PGR-dependent promoters and to be active in a variety of cell types in which PGR-A is inactive. Both isoforms of PGR are differentially expressed in human ESCs. PGR-A may function as the predominant PGR in decidualization [16, 17], although it has also been reported that PGR-B regulates a substantially larger cistrome and transcriptome than PGR-A during ESC differentiation [18]. Generally, the activated PGR directly binds DNA at a specific element in the promoter regions and promotes or represses the transcription of target genes. There are PGR response elements in the promoter regions of the decidual markers PRL and IGFBP-1 [16, 17]. PGRs can also indirectly bind to DNA by interacting with other factors, including CCAAT enhancer-binding proteins (C/EBPs), forkhead box-O1 (FOXO1) transcriptional factors and the signal transducer and activator of transcription 5 (STAT5), which are crucial transcription factors for decidualization [3, 19–22] (Fig. 1B).

## The Link between P4 and cAMP Signaling in Decidualization

Enhanced and sustained levels of intracellular cAMP in P4-primed ESCs imply that the cAMP signaling pathway is activated during P4-dependent decidualization [10]. Medroxyprogesterone and estradiol upregulate the PGE2 receptor, EP2, and PGE2 stimulates the steroid-induced decidualization, suggesting that PGE2 may contribute to an EP2-mediated increase in cAMP levels during P4-stimulated decidualization. Furthermore, Matsuoka *et al.* [23] revealed that Wnt5a, which is secreted by P4-primed human ESCs, activates its Gs-coupled receptor, which in turn stimulates cAMP formation and transcription of super oxide dismutase via cAMP-dependent signaling (Fig. 1B). Although the precise mechanism by which P4 upregulates Wnt5a in ESCs has not been explored, the study of Matsuoka *et al*. [23] has provided new evidence in support of the involvement of P4 in cAMP production in the processes including decidualization (Fig. 1B).

cAMP signaling can integrate P4 action by sensitizing ESCs to P4 and stimulating the transcriptional activity of PGR. Activation of the cAMP signaling pathway reduces the interaction of PGR with the co-repressors, nuclear receptor corepressor (NCoR) and the silencing mediator for retinoid and thyroid hormone receptor (SMRT) [24]. Furthermore, PGR is subject to post-translational sumoylation upon activation, which generally results in repressive properties. cAMP sensitizes ESCs to P4, at least in part, by attenuating the ligand-dependent sumoylation of PGR [25]. Interaction of PGR and a cAMP-regulated transcription factor including FOXO1 and C/EBP*β*, is one of the crucial processes in the induction of decidual markers in human ESCs during decidualization. Thus, P4/PGR and cAMP signaling are involved in cooperative crosstalk during the process of decidualization.

## cAMP Signaling in Decidualization

The intracellular second messenger, cAMP, produced by the stimulation of Gs protein-coupled receptors, regulates a multitude of cellular responses and orchestrates a network of intracellular events. The intracellular cAMP level is controlled by two different types of enzymes, adenylyl cyclases (ACs) and phosphodiesterases (PDEs), which produce and degrade cAMP, respectively. As noted above, cAMP signaling is essential for the initiation of decidualization [4]. In fact, the activity of ACs that

catalyze the cyclization of adenosine triphosphate (ATP) into cAMP is abundant in the endometrium [26]. The endometrial cAMP level is higher during the secretory phase than that during the proliferative phase [27]. P4 or various other bioactive substances that activate the Gs-coupled receptor augment the increase in intracellular cAMP levels in ESCs. Furthermore, forskolin, a chemical compound that activates ACs, also leads to decidualization *in vitro* [11]. In addition to the production of cAMP, the intracellular cAMP level is generally controlled by specific PDEs that catalyze the hydrolysis of cAMP and cyclic guanosine monophosphate into their inactive forms [28]. Among the PDE isozyme families, pharmacological inhibition of PDE4 increases intracellular cAMP levels and decidual marker expression in relaxin-primed human ESCs, indicating the significance of PDE4 as a potential target for pharmacological intervention in decidualization in subfertile women [29].

Various transcription factors that are regulated by the cAMP signaling pathway during decidualization, including C/EBPs, FOXO1 and cAMP response element-binding protein (CREB), play central roles in the expression of the decidual markers PRL and IGFBP-1 in human ESCs (Fig. 1B).

The C/EBPs belong to the leucine zipper family of transcriptional factors. Among the various C/EBPs, abrogation of C/EBP*β* in female mice results in an infertile phenotype due to defective decidualization [30]. C/EBP*β* is predominantly expressed in decidualizing ESCs in the human endometrium and is upregulated by the activation of cAMP signaling [31]. C/EBP binding sites in the decidual PRL and IGFBP-1 promoter regions are essential for cAMP-induced promoter activation [20, 31]. Several lines of evidence suggest that epigenetic regulation, including histone modification, is involved in cAMP-induced PRL and IGFBP-1 expression [32, 33]. C/EBP*β* regulates PRL and IGFBP-1 expression by altering the histone acetylation status of their promoter in human ESCs during decidualization [33]. These results clearly suggest that C/EBP*β* plays a key role in decidualization.

FOXO proteins function as downstream mediators of the phosphoinositol-3-kinase (PI3K)/AKT pathway and modulate the expression of genes involved in cell differentiation, resistance to oxidative stress, DNA damage repair, cell cycle arrest and apoptosis [34]. Post-translational modification, like phosphorylation of FOXO proteins, is the principal mechanism used to regulate various genes. FOXO1, also known as FKHR, is a member of the forkhead family of proteins. It is one of the earliest genes induced in response to cAMP during decidualization [3, 35]. FOXO1 protein accumulates in the nuclei of decidualized cells *in vivo*. Interactions of FOXO1 with PGR and HOXA10 or C/EBP*β* have been shown to induce IGFBP-1 and PRL, respectively, by binding to the promoter regions of these decidual markers [3, 22].

## Protein Kinase a (PKA)-mediated cAMP Signaling in Decidualization

Protein kinase A (PKA) is a cytoplasmic enzyme composed of two regulatory subunits and a catalytic subunit and is well-known as a mediator of cAMP signaling. The catalytic subunit, which is released upon the binding of two cAMP molecules by each regulatory subunit, phosphorylates nuclear target proteins such as CREB and cAMP response element modulator protein (CREM) [36]. These proteins bind to cAMP response elements (CREs) in the control regions of cAMP-responsive genes and modulate their transcription. No changes in the levels of PKA subunit isoform transcripts were detected during decidualization *in vitro*, although a decrease in the protein level of a regulatory subunit isoform was observed. The protein levels of all other forms remained unchanged. This reduction in the level of regulatory subunits may result in a net increase in the activity of free catalytic subunits [4].

Decidual PRL (dPRL) gene transcription is driven by an alternative upstream promoter located approximately 6 kb upstream of the putative transcription start site of an additional, non-coding exon 1A [36]. In human ESCs, cAMP activates the dPRL promoter in a biphasic manner, with an initial weak induction within 12 h, followed by a much more intense induction later [37]. Mutation of the CREs abolished the initial response, but not the later response, suggesting that activation of the cAMP/PKA/CREB pathway stimulates early dPRL induction. Furthermore, the cAMP/PKA pathway induces the dPRL promoter in a delayed fashion through two overlapping C/EBP consensus sequences. As for IGFBP-1, deletion and mutation analysis showed that the CRE in the IGFBP-1 promoter is essential for its activation [38].

## Exchange Protein Directly Activated by cAMP (EPAC)-mediated cAMP Signaling in Decidualization

Exchange protein directly activated by cAMP (EPAC) is a cAMP-activated signaling factor that functions as part of a pathway that is distinct from the classical cAMP/PKA-signaling pathway [39, 40]. There are two isoforms of EPAC, EPAC1 (also known as RAPGEF3) and EPAC2 (RAPGEF4). These proteins share extensive sequence

homology, but are the products of distinct genes in mammals. The structural difference between EPAC1 and EPAC2 is the presence of an additional cyclic nucleotide-binding domain within the N-terminus of EPAC2 [41]. Both EPAC1 and EPAC2 function as guanine nucleotide exchange factors for the Ras family of small guanine triphosphatases (GTPases), including Rap1 [42]. Rap1 and other Rap family members are involved in the regulation of cell proliferation, differentiation, apoptosis and adhesion. Binding of cAMP to the cyclic nucleotide-binding domain of EPAC leads to a conformational change that allows recruitment of Rap protein to a specific domain of EPAC and the conversion of the guanosine diphosphate (GDP)-binding inactive form of Rap to the GTP-binding active form. Thus, Rap1 is a downstream factor in the EPAC signaling pathway. The development of EPAC-selective and PKA-selective cAMP analogs potentially opens a pathway toward understanding the difference between EPAC-mediated and classical PKA-mediated cAMP signaling.

In the human endometrium, constitutive EPAC1 and EPAC2 expression is observed in the epithelial and stromal cells during the proliferative and secretory phases of the menstrual cycle [43]. *In vitro* studies have demonstrated that simultaneous exposure of primary cultured human ESCs or ESC cell lines to the EPAC-selective cAMP analog and the PKA-selective cAMP analog markedly upregulates the expression of IGFBP-1 and PRL, but not that of FOXO1 [43]. However, the EPAC-selective cAMP analog alone does not induce IGFBP-1 and PRL expression. Silencing of EPAC1 or EPAC2 represses cAMP analog-induced IGFBP-1 and PRL expression. EPAC potentiates IGFBP-1 and PRL expression only under conditions of PKA activation [43]. These results imply there is cooperative functioning of the EPAC- and PKA-mediated cAMP signaling pathways in the process of decidualization (Fig. 1B). In addition, we have also demonstrated that EPAC signaling is associated with the differentiation of placental cytotrophoblasts into syncytiotrophoblasts [44].

Conversely, because FOXO1 expression induced by the PKA-selective cAMP analog is not affected by the EPAC-selective cAMP analog, cAMP-dependent FOXO1 expression is predominantly regulated by PKA signaling. Thus, cAMP-responsive gene expression in decidualizing ESCs is differentially and cooperatively regulated by the PKA and EPAC signaling pathways. Our data begs the question; How does EPAC signaling synergize with PKA signaling in the decidualization induced by cAMP analogs? Since the EPAC-selective cAMP analog does not affect the PKA-selective cAMP analog-stimulated phosphorylation of CREB [43], a possible answer is that EPAC signaling may indirectly influence PKA signaling independent of classical CREB phosphorylation. A recent study indicated that the silencing of EPAC1 or EPAC2 downregulates C/EBP*β* mRNA expression, and that the activation of EPAC signaling may upregulate the DNA-binding activity of C/EBP*β* (unpublished data).

Rap1, a downstream signaling factor in the EPAC-mediated cAMP signaling pathway, is activated by EPAC-selective cAMP analog or forskolin in ESCs primed with PKA-selective cAMP analog, but not in non-primed undifferentiating ESCs. This activation is inhibited by knockdown of EPAC1 or EPAC2. Silencing of Rap1 also represses cAMP-induced IGFBP-1 and PRL expression. These results suggest that EPAC/Rap1 signaling is actually activated in decidualizing cells, and that this signaling plays a key role in the acquisition of a secretory phenotype, with IGFBP-1 and PRL, during the process of decidualization induced by the activation of PKA signaling (Fig. 1B).

Recently, we identified calreticulin (CRT) as a potential target of EPAC2, because CRT expression is significantly downregulated in EPAC2-silenced ESCs [45]. CRT is a molecular chaperone that plays a central role in the quality control of newly produced proteins and acts as a regulator of intracellular calcium ion homeostasis [46]. Knockdown of CRT expression in cultured ESCs significantly inhibits cAMP analog- and ovarian steroid-stimulated PRL and IGFBP1 expression as well as morphological differentiation. Thus, EPAC2-mediated CRT expression is essential for decidualization. Silencing of EPAC2 or CRT induces an aberrant, senescence-like phenotype with increased senescence associated-*β*-galactosidase (SA-*β*-Gal) activity and p21 expression as well as decreased p53 expression. Uterine-specific p53 conditional knockout mice display senescence-associated growth restriction with increased SA-*β*-gal activity and p21 expression in the decidua, as well as impaired decidualization and preterm labor [47]. Therefore, it is conceivable that dysregulation of EPAC2 or CRT may impair decidualization, in part, through the induction of abnormal cellular senescence (Fig. 1B).

## Conclusion

Numerous findings have revealed that cAMP signaling regulates endometrial function through the maintenance of cellular homeostasis and the secretion of various hormones and growth factors. EPAC signaling may be essential for the functional and morphological differentiation of ESCs into decidual cells. Further study of the

functions of EPAC in uterine physiology will provide novel data that can be used to explore the regulatory mechanisms of decidualization, implantation and placentation under physiological and pathological conditions. Acquiring such data is an important step toward a better understanding of the mechanisms that regulate reproduction in females. From the point of view of clinical investigators, the issues we should address include: infertility caused by the disturbance of the physiological activity of EPAC during the early secretory phase of the menstrual cycle, and the development of novel targeted drugs that enhance the coordinated regulation of cAMP signals toward decidualization. Furthermore, future studies should aim determine: 1) the precise relationship between the EPAC signaling pathway and other pathways, including the P4/PGR and cAMP/PKA signaling pathways as well as the function of CRT in various reproductive processes; 2) the function of EPAC and epigenetic regulation, such as DNA methylation and acetylation, that modulate the expression of genes associated with implantation and decidualization; and 3) the transcriptome and metabolomic profiles of changes governed by EPAC or PKA signaling.

## Acknowledgments

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.

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