REVIEW

Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair

B Gellersen and **J Brosens**[1]

IHF Institute for Hormone and Fertility Research, University of Hamburg, Hamburg, Germany

[1]Institute of Reproductive and Developmental Biology, Wolfson and Weston Research Centre for Family Health, Imperial College London, Hammersmith Campus, London W12 0NN, UK

(Requests for offprints should be addressed to J Brosens; Email: j.brosens@ic.ac.uk)

(B Gellersen is currently at Endokrinologikum Hamburg 20251 Hamburg, Germany)

Abstract

During the menstrual cycle, the ovarian hormones oestradiol and progesterone control the ordered growth and differentiation of uterine cells. This remodelling process is critical for implantation of the developing embryo, the formation of the placenta, and maintenance of pregnancy. Failure of uterine tissues to respond appropriately to ovarian hormone signalling results in defective placentation, associated with a spectrum of pregnancy disorders such as recurrent miscarriages and preeclampsia. These obstetrical disorders are a major cause of maternal and perinatal morbidity and mortality. Progesterone exerts its action on target cells, at least in part, through binding to the progesterone receptor (PR), a member of the steroid/thyroid hormone receptor superfamily of ligand-activated transcription factors. The mechanism by which progesterone controls the differentiation of human endometrial stromal cells, a process termed decidualization, in the secretory phase of the menstrual cycle is not well understood. Emerging evidence indicates that locally expressed factors and activation of the cAMP second messenger pathway integrate hormonal inputs and confer cellular specificity to progesterone action through the induction of diverse transcription factors capable of modulating PR function.

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Introduction

The postovulatory rise in ovarian progesterone induces profound remodelling of the oestrogen-primed endometrium, characterized initially by growth and coiling of the spiral arteries, secretory transformation of the glands, influx of distinct immune cells, and subsequently by decidualization of the stromal compartment. Decidualization represents a process of morphological and biochemical differentiation (Fig. 1). The decidualized endometrial stromal cell (ESC) becomes rounded, acquires myofibroblast characteristics, and secretes a variety of phenotypic antigens, including prolactin (PRL), insulin-like growth factor binding protein-1 (IGFBP-1) and tissue factor (TF) (Daly *et al.* 1983, Irwin *et al.* 1989, Tabanelli *et al.* 1992, Tseng *et al.* 1992, Lockwood *et al.* 1993, Gellersen *et al.* 1994, Oliver *et al.* 1999*b*, Christian *et al.* 2001*b*). At a molecular level, decidual transformation involves extensive reprogramming of many cell functions including altered steroid hormone receptor expression and steroid metabolism, remodelling of the extracellular matrix and cytoskeleton, altered expression of intracellular enzymes, growth factors and cytokines and their receptors, and induction of apoptosis modulators and decidua-specific transcription factors (Oliver *et al.* 1999*a*, Popovici *et al.* 2000, Brar *et al.* 2001).

The term 'decidualization' is derived from the Latin verb 'decidere' which means to 'fall off'. In the 19th century, Thomas Huxley suggested that the order Mammalia should be subdivided into 'Deciduata' and 'Adeciduata', depending on whether the uterine mucosa is cast off at parturition. In contrast to many species, decidualization of the endometrial stroma in humans is independent of the presence of an implanting blastocyst. However, in the absence of conception, falling circulating progesterone levels in the late secretory phase of the cycle elicit sloughing of the decidualized superficial endometrial layer and menstruation. In pregnancy, the decidual reaction extends to the basal endometrial layer and is critical for trophoblast invasion and placenta formation (Brosens *et al.*

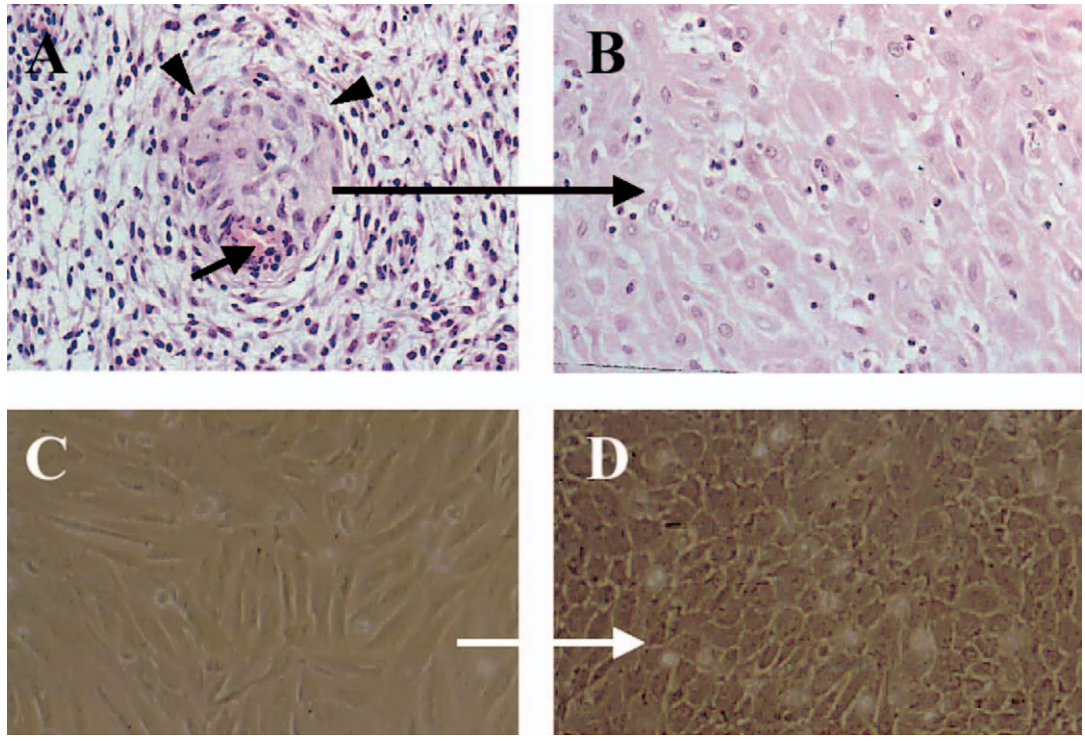

Figure 1 Decidual transformation of endometrial stromal cells (ESC) *in vivo* and *in vitro*. (A) Initiation of the decidual response in stromal cells (arrow heads) on day 23 of the cycle around the terminal portion of a spiral artery (arrow). (B) Extensive decidual transformation of the stromal compartment of the superficial endometrium in the late luteal phase of the cycle. (C) Undifferentiated primary ESC display a fibroblastic spindle-shaped morphology. (D) Treatment of confluent monolayers with 8-bromo-cAMP, alone or in combination with a progestin, for 48 h transforms the spindle-shaped cells into cells with larger nuclei and abundant cytoplasm, resembling decidual cells.

2002). Indeed, decidualization only occurs in species in which placentation involves breaching of the luminal epithelium by the trophoblast and the extent of this differentiation process often correlates with the degree of trophoblast invasion (Ramsey *et al.* 1976, Brosens *et al.* 2002). Furthermore, various mouse knock-out models have provided unequivocal proof that endometrial decidualization is essential for maintaining pregnancy (Table 1). Implantation and early pregnancy are further characterized by profound vascular changes and influx of uterine natural killer cells and macrophages (King 2000, Brosens *et al.* 2002). Hence, it appears likely that the decidual reaction *per se* is important to protect endometrial cells against inflammatory signals and oxidative stress.

Table 1 Knock-out mice with impaired decidual reaction

	Reference
Disrupted gene	
COX-2	Lim *et al.* (1997)
Leukaemia inhibitory factor	Stewart & Cullinan (1997)
Progesterone receptor	Lydon *et al.* (1995)
PR-A	Mulac-Jericevic *et al.* (2000)
Steroid receptor coactivator-1	Xu *et al.* (1998)
Hoxa-11	Gendron *et al.* (1997)
Hoxa-10	Ma *et al.* (1998)
IL-11 receptor	Bilinski *et al.* (1998), Robb *et al.* (1998)
Components of IFN-γ signalling	Ashkar *et al.* (2000)

There is abundant clinical and experimental evidence in support of a critical role of progesterone in maintaining the decidual phenotype. However, decidual transformation is first apparent in stromal cells surrounding the spiral arteries approximately ten days after the postovulatory rise in ovarian progesterone levels (de Ziegler *et al.* 1998), indicating that the expression of decidua-specific genes is unlikely to be under direct transcriptional control of activated progesterone receptor (PR). Furthermore, progesterone is a very weak inducer of the decidual phenotype in cultured purified primary ESC (Fig. 2). Evidence has emerged to suggest that initiation of the decidual process requires elevated intracellular cAMP levels and sustained activation of the protein kinase A (PKA) pathway. This commentary focuses on the intricacies of cAMP and PR signalling and cross-talk in human ESC which hitherto have escaped recognition in model cell lines.

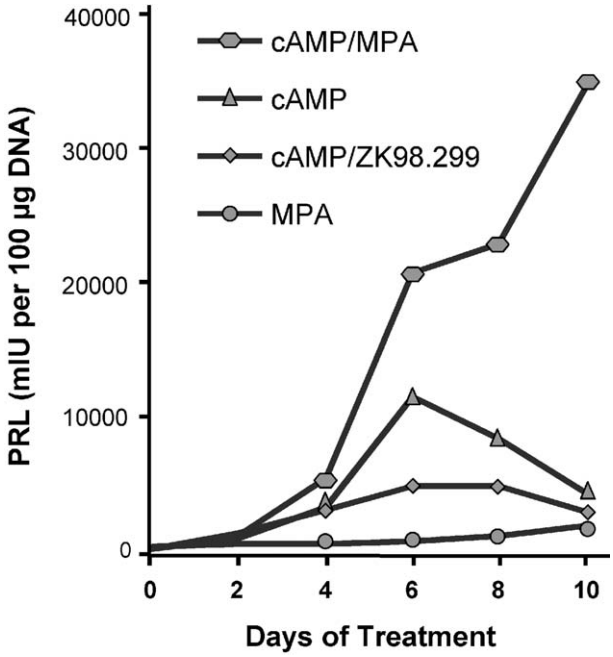

Figure 2 PRL production by primary ESC in response to cAMP, progestins and antiprogestins. Confluent ESC were treated with 8-bromo-cAMP (cAMP; 0·5 mM), the progestin MPA (10^{-6} M), the pure antiprogestin ZK98.299 (10^{-6} M), or a combination of these as indicated. The medium was changed every 48 h. The data represent the mean of PRL concentrations in the supernatant, normalized for DNA content in each well at a given time point.

Biochemical mechanism of decidualization: role of cyclic AMP

Cyclic AMP signal transduction – an overview

Cyclic AMP is a ubiquitous second messenger molecule which is generated upon binding of a ligand to members of a receptor family which are classified by seven transmembrane-spanning domains. These receptors are coupled to the heterotrimeric guanine nucleotide-binding proteins (G-proteins) and are hence designated G-protein-coupled receptors (GPCR). Upon ligand binding, the Gα subunit is released from the trimeric Gαβγ complex and regulates the activity of adenylyl cyclase, an enzyme which produces cAMP from ATP (Dessauer *et al.* 1996). A major downstream recipient of cAMP is the cAMP-dependent PKA, a cytoplasmic enzyme which in its basal state is composed of two regulatory and two catalytic subunits (Skålhegg & Taskén 2000). Upon binding of two cAMP molecules to each regulatory subunit, the latter undergo a conformational change which results in release and activation of the catalytic subunits. These may phosphorylate target molecules in the cytoplasm or diffuse into the nucleus and modulate the activity of transcription factors by phosphorylation. Major nuclear targets of PKA phosphorylation are the cAMP response element binding protein (CREB) and the related cAMP response element modulator (CREM) (Mayr & Montminy 2001). CREB and CREM belong to the family of basic region/leucine zipper (bZIP) transcriptional regulators which dimerize through the leucine zipper and bind to their cognate DNA sequence through the basic region (Luscombe *et al.* 2000). The optimal binding site for CREB and CREM is the palindromic cAMP response element (CRE) TGACGTCA. Phosphorylated CREB/CREM recruits the co-activator CREB binding protein (CBP) to the promoter region of their target genes. CBP, owing to its inherent histone acetyltransferase activity, facilitates transcription by modulation of chromatin conformation (Ogryzko *et al.* 1996, Montminy 1997, Mayr & Montminy 2001).

Cyclic AMP signal transduction in differentiating human endometrium

Cyclic AMP signalling in general is controlled at many levels. These include regulation at the receptor level, catabolism of cAMP by phosphodiesterases, modified composition of the PKA holoenzyme, expression of CREB and CREM isoforms with altered transcriptional activity, or a change in the expression level of coactivators or corepressors. Ultimately, in most cell systems, these mechanisms are aimed at terminating cellular responses to a lasting external stimulus. Human endometrial stromal cells represent an exception to this rule in that they are dependent on a persistent stimulation of the cAMP pathway to acquire and maintain the decidualized phenotype (Tanaka *et al.* 1993, Telgmann & Gellersen 1998). Upon withdrawal of the cAMP stimulus, decidualized ESCs re-acquire an undifferentiated phenotype and cease to express differentiation markers such as PRL and IGFBP-1 (authors' unpublished observations).

G-protein-coupled receptors Peptide hormones and prostanoids implicated in promoting the decidual transformation include the gonadotrophins luteinizing hormone/human chorionic gonadotrophin (LH/hCG), corticotrophin releasing hormone (CRH), relaxin (RLX), and prostaglandin E_2 (PGE_2) (Tseng *et al.* 1992, Tang & Gurpide 1993, Frank *et al.* 1994, Ferrari *et al.* 1995). These molecules share the ability to provoke an increase in intracellular cAMP levels by binding to GPCR coupled to the stimulatory Gsα protein (Gilchrist *et al.* 1996, Herrlich *et al.* 1996, Gravanis *et al.* 1999, Narumiya *et al.* 1999, Breyer *et al.* 2001, Narumiya & FitzGerald 2001, Hillhouse & Grammatopoulos 2002, Hsu *et al.* 2002, Sudo *et al.* 2002). While the presence of gonadotrophin receptors on cultured ESC has been demonstrated (Han *et al.* 1997), the decidualizing potential of LH/hCG is still a matter of debate (Tang & Gurpide 1993, Kasahara *et al.* 2001). The PGE_2 receptor subtypes EP_2 and EP_4 and the

mRNA for the CRH receptor R1 have been detected in nonpregnant human endometrium across the menstrual cycle (Di Blasio *et al.* 1997, Milne *et al.* 2001). The nature of RLX binding sites has long been an enigma. Only recently have two orphan receptors, LGR7 and LGR8, been shown to serve as RLX receptors (Hsu *et al.* 2002, Sudo *et al.* 2002). LGR7 and LGR8 belong to the subgroup of leucine-rich repeat-containing G-protein-coupled receptors (as do the gonadotrophin and thyrotrophin receptors) characterized by a long extracellular domain. Although interaction of RLX with these receptors clearly results in accumulation of cAMP, the signalling pathway does not appear solely to involve activation of adenylyl cyclase by the Gsα subunit. Intriguingly, RLX-induced cAMP production can be blocked by tyrosine kinase inhibitors (Bartsch *et al.* 2001). Incubation of ESC with RLX leads to rapid phosphorylation of the mitogen activated protein kinase (MAPK) kinase, MEK, followed by phosphorylation of MAPK. Subsequently, CREB is phosphorylated on Ser-133, the same site that is the target of phosphorylation by PKA (Zhang *et al.* 2002). In this context, it should be noted that in recent years new paradigms have emerged which go beyond the classical view of GPCR signalling being solely effected by G-proteins. Ligand-activated GPCR can associate with a variety of intracellular proteins including arrestins, G-protein-coupled receptor kinases (GRK), or proteins containing SH3 domains. These interactions couple the receptors to diverse intracellular signalling pathways (Hall *et al.* 1999, Marinissen & Gutkind 2001).

Phosphodiesterases It is not only the rate of synthesis that determines the intracellular level of cAMP, but also its degradation. Members of the large family of phosphodiesterases convert cAMP to AMP which no longer stimulates PKA activity (Mehats *et al.* 2002). It is tempting to speculate that the sustained increase in cellular cAMP observed in decidualizing ESC is, at least in part, due to inhibition of phosphodiesterase activity. This may be brought about by the proposed coupling of RLX receptor signalling to the tyrosine kinase pathway and inhibition of phosphodiesterase activity by tyrosine phosphorylation (Bartsch *et al.* 2001, Ivell 2002).

PKA composition An important determinant of PKA activity and subcellular localization is the composition of the holoenzmye (Skålhegg & Taskén 2000). Four isoforms of the regulatory subunit (RIα, RIβ, RIIα, RIIβ) and of the catalytic subunit (Cα, Cβ, Cγ, PrKX) have been described (Taskén *et al.* 1997). Decidualizing treatment of ESC with RLX leads to a marked and specific down-regulation of RIα protein, whereas the levels of RIβ, RIIα, RIIβ and of the C-subunits remain unchanged (Telgmann *et al.* 1997). As a consequence of reduced total R-subunit levels, the R:C ratio is shifted towards the C-subunits, presumably resulting in a net increase in free, activated C protein and an increase in target protein phosphorylation.

CREM isoform expression Among the nuclear targets of PKA C-subunit are CREB and CREM. Their core region is a bipartite transactivation domain, consisting of one or two glutamine-rich regions (Q1 and Q2), and the central kinase-inducible domain (KID) harbouring the phosphorylation sites (Mayr & Montminy 2001). Due to alternative splicing, alternative translation initiaton events, or alternative promoter usage, these transcription factors can be expressed in a multitude of isoforms (Walker & Habener 1996, Gellersen *et al.* 1997, 2002). Depending on the presence or absence of constituents of the transactivation domain, these isoforms are transcriptional activators or repressors (Mayr & Montminy 2001). While the expression of CREB is largely constitutive in many systems, its action being tightly regulated by phosphorylation and de-phosphorylation events, the *CREM* gene carries an internal, highly cAMP-inducible promoter P2 (Molina *et al.* 1993). Transcripts generated from P2 encode the C-terminal bZIP region but are devoid of the N-terminal transactivation functions. The translation product is known as ICER (inducible cAMP early repressor); through homodimerization or heterodimerization with other CREM/CREB isoforms it functions as a potent repressor and establishes a negative feedback loop to down-regulate transcription of cAMP-induced promoters including its own. By this mechanism a cAMP-mediated signal is terminated (Foulkes *et al.* 1996). However, ESC again represent an exception to this concept. When exposed to long-term treatment with RLX or cAMP analogue, they do not show the expected transient increase in ICER expression but a persistent upregulation of ICER, indicating a permissiveness of the cells to the ongoing stimulation of cAMP signalling (Gellersen *et al.* 1997).

Coactivators The transcriptional coactivator CBP (or its paralogue p300) had originally been identified based on its ability to bind to CREB (Chrivia *et al.* 1993). It is now recognized as an integrator for a large number of transcriptional signals, owing to its interaction with transcription factors of surprisingly diverse nature on the one hand and RNA polymerase II complexes on the other hand, thus establishing contact between specific inputs and the basal transcription machinery (Janknecht & Hunter 1996, Kamei *et al.* 1996). It is known to interact with, and enhance the activity of, CCAAT/enhancer-binding protein β (C/EBPβ) (Mink *et al.* 1997) which is an important mediator of cAMP signalling in ESC, as will be outlined below (Pohnke *et al.* 1999). Furthermore, CBP is recruited to preinitiation complexes containing steroid hormone receptors through the 160 kDa steroid receptor co-activator proteins including SRC-1 (Smith *et al.* 1996). To date, our knowledge of the expression profiles of co-activators and corepressors, and of their potential

hormone-dependency, in human endometrium is very limited. At least the presence of SRC-1 has been demonstrated in endometrial stromal cells throughout the menstrual cycle, and in cultured ESC (Brosens *et al.* 1999, Gregory *et al.* 2002, Wieser *et al.* 2002).

Novel cAMP-binding proteins Recently, novel cAMP-binding proteins have been identified, the cAMP-guanine nucleotide exchange factors or EPACs (exchange protein activated by cAMP). These mediate PKA-independent signal transduction and couple the cAMP pathway to the p38 MAPK and phosphatidylinositol 3-kinase (PI3K) signalling cascades (Richards 2001). Notably, EPAC and PKA can mediate opposing effects of cAMP on downstream targets (Mei *et al.* 2002). The role of EPACs in mediating the cAMP responses in human endometrium remains to be determined. However, treatment of undifferentiated primary ESC with 8-p CPT-2′-*O*-methyl-cAMP, a novel EPAC-specific cAMP analoque (Enserink *et al.* 2002), fails to elicit a decidual phenotype, suggesting that cAMP-dependent differentiation is predominantly or exclusively mediated through activation of PKA (authors' unpublished observations).

Downstream events of cAMP signalling in ESC: analysis of the decidual PRL promoter

The onset of PRL production *in vivo* in endometrial stromal cells of the late secretory phase is recapitulated in cultured ESC when they are exposed to the appropriate decidualizing stimuli (Daly *et al.* 1983, Irwin *et al.* 1989, Tabanelli *et al.* 1992, Lane *et al.* 1994). Activation of the hitherto silent PRL gene in decidualizing cells serves as an exquisite marker of differentiation (Christian *et al.* 2001*a*). It has to be noted that the PRL gene in the decidua is transcribed from an alternative promoter located 6 kb upstream of the pituitary PRL promoter and therefore underlies completely different regulatory mechanisms (Berwaer *et al.* 1994, Gellersen *et al.* 1994). Transcription from the decidual PRL (dPRL) promoter adds a non-coding exon (exon 1a) to the PRL mRNA, the resulting protein, however, is identical to that produced in the pituitary (DiMattia *et al.* 1990). Utilization of an alternative decidua-specific PRL promoter has so far only been demonstrated in humans and primates, which excludes *in vivo* and *in vitro* experimentation in rodent models for understanding this aspect of decidualization (Brown & Bethea 1994, Frasor *et al.* 1999).

CCAAT/enhancer-binding protein β (C/EBPβ) We have exploited the dPRL promoter as a tool to identify transcription factors relevant to decidualization. Just as morphological decidualization of cultured ESC is achieved within 2–4 days of treatment with a cAMP analogue, dPRL mRNA expression and PRL secretion become detectable within this time frame (Fig. 2) (Telgmann *et al.* 1997, Telgmann & Gellersen 1998). In order to investigate whether this induction is due to transcriptional activation, we performed transient transfection experiments in primary ESC and observed a biphasic pattern of cAMP-mediated induction of the dPRL promoter. Whereas a CRE-like sequence in close proximity to the transcriptional start site conferred a rapid but weak and transient induction on the promoter, a delayed but strong and persistent stimulation of promoter activity was mediated by the region −332/−270 relative to the start site (Telgmann *et al.* 1997). The time course of induction, and the lack of CRE-like sequences within this region suggested an indirect mode of activation. The element −332/−270 contains two binding sites for C/EBPs which constitute another subgroup of bZIP transcription factors (Ramji & Foka 2002). Among the various members of this group, C/EBPβ is the predominant form in decidualized stromal cells (Pohnke *et al.* 1999). Of note, C/EBPβ has been recognized as essential for female reproduction; its absence obliterates ovulation, breast development and function (Sterneck *et al.* 1997, Robinson *et al.* 1998, Seagroves *et al.* 1998). The C/EBP binding sites in the dPRL promoter were shown to be crucial for cAMP-induced activation, revealing C/EBPβ as a central mediator of the cAMP signal towards decidualization (Pohnke *et al.* 1999). Not only is C/EBPβ induced by cAMP in cultured ESC, it also shows a striking increase in expression *in vivo* in stromal cell nuclei of the late secretory phase (Christian *et al.* 2002*b*). There are two isoforms of C/EBPβ, the full-length liver-enriched activating protein (LAP) and the truncated liver-enriched inhibitory protein (LIP). The latter lacks the N-terminal transactivation domains of LAP and acts as a potent repressor of C/EBP-dependent transcription (Descombes & Schibler 1991). Western blot analysis studies showed that only LAP is present in normal non-pregnant human endometrium (Christian *et al.* 2002*b*).

Forkhead proteins In addition to binding to their cognate DNA sequences as homo- or heterodimers, C/EBPs have been shown to engage in protein–protein interactions with a wide variety of nuclear proteins. Investigating C/EBPβ-mediated activation of the dPRL promoter further, we identified a member of the family of forkhead/winged helix proteins (Burgering & Kops 2002), FoxO1a (FKHR), as a novel interacting partner (Christian *et al.* 2002*b*). FoxO1a and the other members of the FoxO subclass of forkhead transcription factors FoxO4 (AFX) and FoxO3a (FKHRL1) (Kaestner *et al.* 2000) have been shown to control the expression of genes essential for metabolic responses, cell cycle regulation and apoptosis, and confer resistance to oxidative stress (Brunet *et al.* 1999, Guo *et al.* 1999, Dijkers *et al.* 2000, Medema *et al.* 2000, Furukawa-Hibi *et al.* 2002, Kops *et al.* 2002*a*,*b*, Scott *et al.* 2002). FoxO1a expression is induced by cAMP in

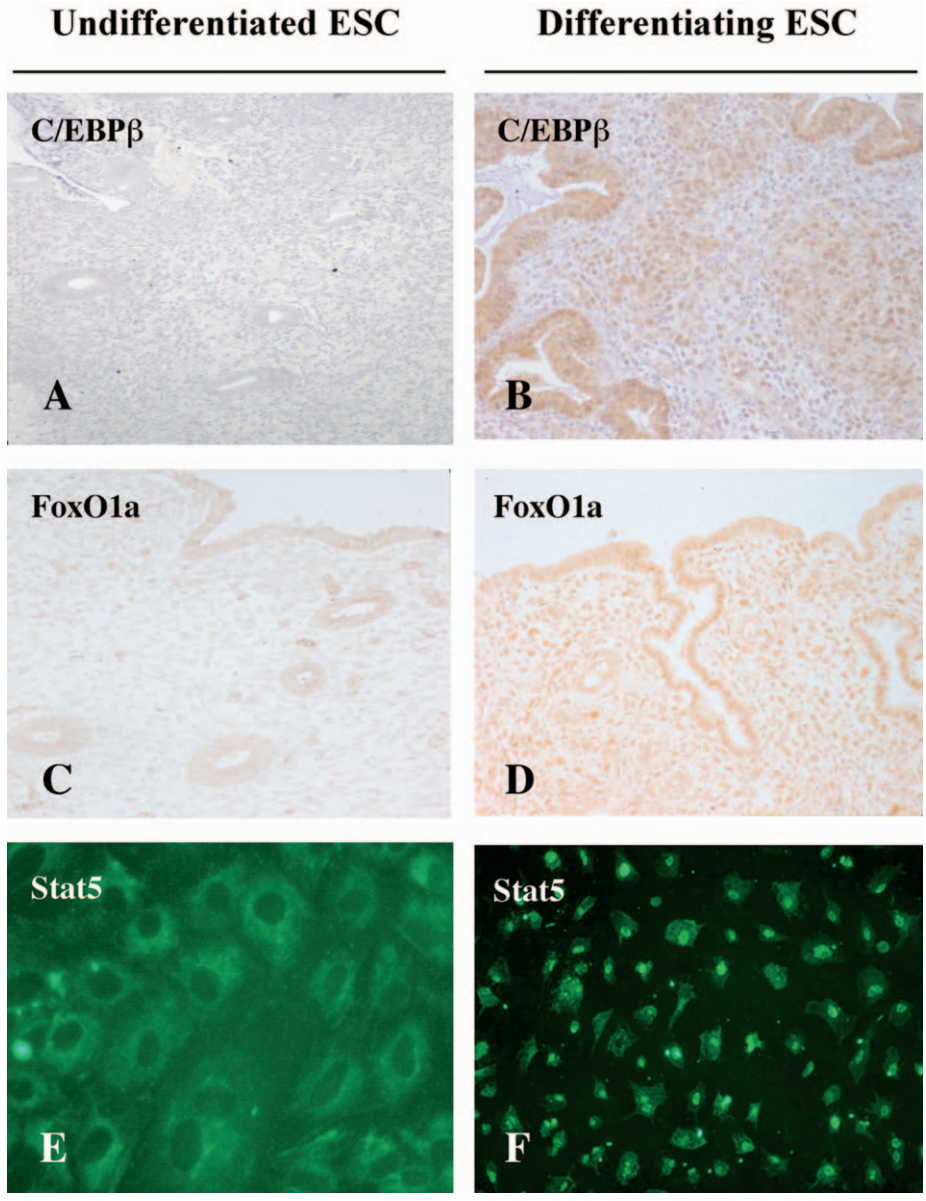

Figure 3 Expression of C/EBPβ, FoxO1a, and Stat5 in differentiating ESC. (A, C) Proliferative phase endometrium shows very weak FoxO1a immunostaining confined to glandular and surface epithelial cells. In contrast, there is no discernible C/EBPβ expression in either glandular or stromal compartments. (B, D) Late secretory phase endometrium showing strong C/EBPβ and FoxO1a expression in stroma and glands. (E) Stat5 expression in undifferentiated ESC in culture is confined to the cytoplasm. (F) Upon treatment with 8-bromo-cAMP and a progestin, Stat5 accumulates in the cell nuclei.

cultured ESC, and, in striking parallelism to C/EBPβ, FoxO1a protein accumulates in the nuclei of decidualized stromal cells *in vivo* (Fig. 3). FoxO1a and C/EBPβ physically interact and cooperatively activate the dPRL promoter (Christian *et al.* 2002*b*). FoxO are targets of protein kinase B (PKB/Akt), a serine/threonine kinase located downstream of PI3K. FoxO1a has three putative PKB/Akt phosphorylation sites (Thr-24, Ser-256, Ser-319) which are also conserved in DAF16, the nematode *Caenorhabditis elegans* homologue. Upon PKB/Akt phosphorylation, DAF16 and its human counterparts are retained in the cytoplasm, and their exlusion from the nucleus is associated with reduced transcriptional activity (Biggs *et al.* 1999, Brunet *et al.* 1999, Rena *et al.* 1999).

The observation that FoxO1a accumulates in the nuclei of cAMP-treated ESC suggests that the PI3K/PKB signalling pathway is suppressed upon decidualization. This is in agreement with *in vivo* studies demonstrating that PTEN, a tumour suppressor gene and potent inhibitor of the PI3K/PKB signalling pathway, is highly expressed in the cytoplasm of stromal cells undergoing decidual transformation during the late secretory phase of the menstrual cycle (Mutter *et al.* 2000).

Signal transducer and activator of transcription 5 (Stat5) A recent study demonstrated that Stat5 also enhances the activity of the −332/−270 dPRL promoter region in human ESC (Mak *et al.* 2002). Stat5 belongs to the STAT family of latent transcription factors which have been implicated in growth and differentiation of many tissues including adipocytes, hepatocytes, and mammary epithelial cells (Darnell 1997). Treatment of primary ESC cultures with cAMP with or without progestin for two or more days results in induction, phosphorylation, dimerization, and nuclear translocation of Stat5 (Fig. 3) (Mak *et al.* 2002). Stats are activated by numerous cytokines and peptide growth factors. They lack intrinsic kinase activity and, in most cell systems, require targeted phosphorylation by receptor-associated Janus kinases (Jaks) for signal transduction (Schindler & Darnell 1995, Darnell 1997). However, Mak and co-workers demonstrated that nuclear accumulation of phospho-Stat5 in ESC is independent of Jak activity, indicating a role for other activating kinases (Mak *et al.* 2002). One such factor capable of activating Stat5 in a Jak-independent manner is c-Src kinase (Olayioye *et al.* 1999), which is highly expressed in differentiating ESC (Maruyama *et al.* 1999, Yamamoto *et al.* 2002).

Biochemical mechanism of decidualization: role of PR

Progesterone signal transduction: role of nuclear PR

Ovarian progesterone is required for all aspects of female reproductive function including sexual behaviour, mammary gland development, ovulation, implantation, and maintenance of pregnancy (Conneely & Lydon 2000, Mulac-Jericevic *et al.* 2000, Rider 2002). Genomic actions of progesterone in target cells are mediated through activation of its nuclear receptor. The progesterone receptor (PR) is a member of the superfamily of ligand-activated transcription factors that exhibit sequence-specific DNA binding to regulatory regions of their target genes. Two isoforms exist, PR-A and PR-B, which arise from different promoter usage in a single gene. PR-B differs from PR-A in that it contains an additional 164 amino acids at the N-terminus (B-upstream sequence, BUS) (Kastner *et al.* 1990). Although the PR isoforms display indistinguishable hormone- and DNA-binding affinities, several studies have shown that, depending on the cell- and promoter context, PR-A and PR-B have remarkably different transcriptional activities. In general, the PR-A isoform is transcriptionally much less active and functions as a dominant inhibitor of transcription by PR-B and various other steroid receptors (Tung *et al.* 1993, Vegeto *et al.* 1993, Sartorius *et al.* 1994*a*, Wen *et al.* 1994). Various models exist to explain the weak transactivation potential of PR-A compared with PR-B. PR-A shares with PR-B the activation functions AF-1 and AF-2 but lacks AF-3, which is situated in the BUS segment specific to PR-B (Sartorius *et al.* 1994*b*). AF-1 is a constitutive activation domain N-terminal to the DNA-binding domain (DBD) while the ligand-dependent activation function AF-2 is located in the ligand-binding domain (LBD) (Meyer *et al.* 1992). The N-terminal segment of PR-A harbours an inhibitory function, termed IF or ID, which represses AF-1 or AF-2, but not AF-3. Removal of IF/ID converts PR-A into a strong transcriptional activator. The BUS domain is thought to repress IF/ID, thereby rendering PR-B a much more potent activator of transcription than PR-A (Hovland *et al.* 1998). Recently a SUMO-1 (small ubiquitin-like modifier-1) binding motif has been identified within the IF domain of PR (Abdel-Hafiz *et al.* 2002). Sumoylation involves covalent binding of SUMO (SUMO-1, SUMO-2, or SUMO-3) to target proteins. Like ubiquitination, sumoylation uses a battery of activating (E1), conjugating (E2), and ligating (E3) enzymes. In contrast to ubiquitination, sumoylation does not lead to protein degradation. Instead, SUMO-1 modification of transcription factors has profound consequences on protein stability, subcellular localization, interactions with other nuclear proteins, and transcriptional activity (Melchior 2000, Hochstrasser 2001, Muller *et al.* 2001). Mutation of the N-terminal SUMO-1 binding motif markedly increases the transcriptional activity of PR-A and PR-B and abolishes the transrepression activity of PR-A (Abdel-Hafiz *et al.* 2002). It has also been reported that the lower transactivation potential of PR-A may be a result of its higher affinity for the corepressor SMRT (silencing mediator of retinoid and thyroid hormone receptor) and its less efficient recruitment of the coactivator SRC-1 (Giangrande *et al.* 2000).

Binding of ligand induces a conformational change in the receptor, resulting in phosphorylation, dissociation from heat shock proteins, dimerization, sumoylation of a subpopulation of the receptor, and binding and activation of specific response elements in the promoter region of target genes. The latter requires further interaction of the AF-2 region with SRCs resulting in recruitment of other SRC-associated histone acetyltransferases (CBP and pCAF) and the methyltransferase CARM1 (Chen *et al.* 1999, Wardell *et al.* 2002). Hormone binding is not an absolute requirement for the activation of steroid receptors. For instance, elevated intracellular cAMP levels and

activation of the PKA pathway can induce ligand-independent activation of chicken PR (cPR), the androgen receptor (AR), and the oestrogen receptor (ER) in certain cell systems (Denner *et al.* 1990, Aronica & Katzenellenbogen 1993, Nazareth & Weigel 1996). To our knowledge, there is no convincing evidence that PKA, or any other signalling pathway, can activate unliganded PR in human reproductive tissues. However, cAMP analogues such as 8-bromo-cAMP enhance hormone-dependent transcriptional activity of PR and can convert some antiprogestins, such as RU486, into PR agonists. The mechanism by which cAMP potentiates PR activity is not entirely understood but is thought to involve disruption of the interaction between the receptor and the corepressors NCoR and SMRT and increased cooperation between coactivators such as SRC-1 and CBP (Wagner *et al.* 1998, Rowan *et al.* 2000*a*,*b*).

Finally, it should be noted that liganded PR can profoundly modulate gene expression through protein–protein interaction with other transcription factors rather than through direct interaction with DNA. In fact, our current evidence indicates that this is the dominant mechanism of PR action in differentiating human ESC (see below).

Progesterone signal transduction: role of membrane PR

Progesterone, like other steroid hormones, can trigger rapid cytoplasmic events that are independent of its genomic actions (Boonyaratanakornkit *et al.* 2001, Cato *et al.* 2002). One of the best characterized biological examples is the resumption of meiosis in *Xenopus* oocytes arrested at the G2 to prophase border in response to progesterone (Maller 2001). It has been suggested that these rapid non-genomic effects of progesterone are mediated by binding of PR to the SH3 domain of c-Src, resulting in phosphorylation and activation of the p42/44 MAPK signal transduction pathway (Boonyaratanakornkit *et al.* 2001). However, very recently a family of membrane progestin receptors (mPR-α, mPR-β, mPR-γ) has been discovered. These novel membrane PRs are structurally distinct from their nuclear counterparts but related to GPCRs (Zhu *et al.* 2003*a*,*b*). The mPR-α, first cloned from sea trout, is thought to be coupled to an inhibitory G protein. This mechanism of action of the mPR-α is consistent with the rapid inhibition of adenylyl cyclase activity observed in progestin-treated *Xenopus* oocytes. Moreover, injection of zebrafish oocytes with zebrafish mPR-α antisense blocked steroid-induced oocyte maturation (Zhu *et al.* 2003*b*).

The human mPR-α is predominantly expressed in reproductive tissues (Zhu *et al.* 2003*a*). No information is as yet available on its role in differentiating ESCs but the fact that mPR-α is present in the uterus and placenta will undoubtedly trigger renewed interest in the impact of progesterone on cytoplasmic signalling events in female reproduction.

Progesterone signalling in ESC: analysis of the decidual PRL promoter

Several lines of evidence support the concept that interaction of nuclear PR with other transcription factors mediates the progesterone effects in differentiating endometrial stroma. First, decidualization, *in vivo* and *in vitro*, is associated with rapid down-regulation of PR-B rendering PR-A the dominant isoform (Wang *et al.* 1998, Brosens *et al.* 1999, Mote *et al.* 1999). The critical role of this receptor isoform in the stroma is further demonstrated by the lack of a decidual response in uteri of PR-A deficient mice (Mulac-Jericevic *et al.* 2000). As mentioned, binding of PR-A to its DNA response element generally elicits a very weak transcriptional response and it appears counterintuitive that this mechanism would suffice to induce the profound cellular reprogramming which has to be maintained throughout pregnancy. Secondly, progestin treatment of primary cultures does elicit modest expression of decidual markers, such as IGFBP-1 and PRL, but only after several days of stimulation by which time the intracellular cAMP levels are increasing (Brar *et al.* 1997). Many decidua-specific genes, including the decidual PRL gene, do not have palindromic progesterone response elements in their promoters. Furthermore, in transient transfection experiments, progestin treatment alone fails to activate the dPRL promoter in the presence or absence of overexpressed PR-A or PR-B (Gellersen *et al.* 1994, Brosens *et al.* 1999, Mak *et al.* 2002). However, progestins markedly enhance dPRL promoter activity, as well as PRL mRNA and protein expression in cells pretreated with cAMP for approximately two days (Brosens *et al.* 1999). These observations suggested that the PKA signalling pathway may sensitize ESC to progesterone through induction or modification of transcription factors or coactivators capable of modulating PR function. This does indeed appear to be the case.

PR and C/EBPβ The presence of a PR binding half-site adjacent to the C/EBP binding sites in the dPRL −332/−270 promoter element prompted us to investigate whether C/EBPβ might tether PR to the dPRL promoter. We demonstrated that PR can physically associate with the two C/EBPβ isoforms, LAP and LIP. This interaction is mediated by the DBD of the receptor and the bZIP domain of C/EBPβ (Christian *et al.* 2002*a*). The functional consequences of this interaction are dependent upon the relative ratios of PR and C/EBPβ isoforms in the cell. Transfection studies demonstrated that PR-A, but not PR-B, greatly enhances LAP-dependent activation of the dPRL −332/−270 promoter region as well as a reporter construct driven by a single C/EBPβ response element in

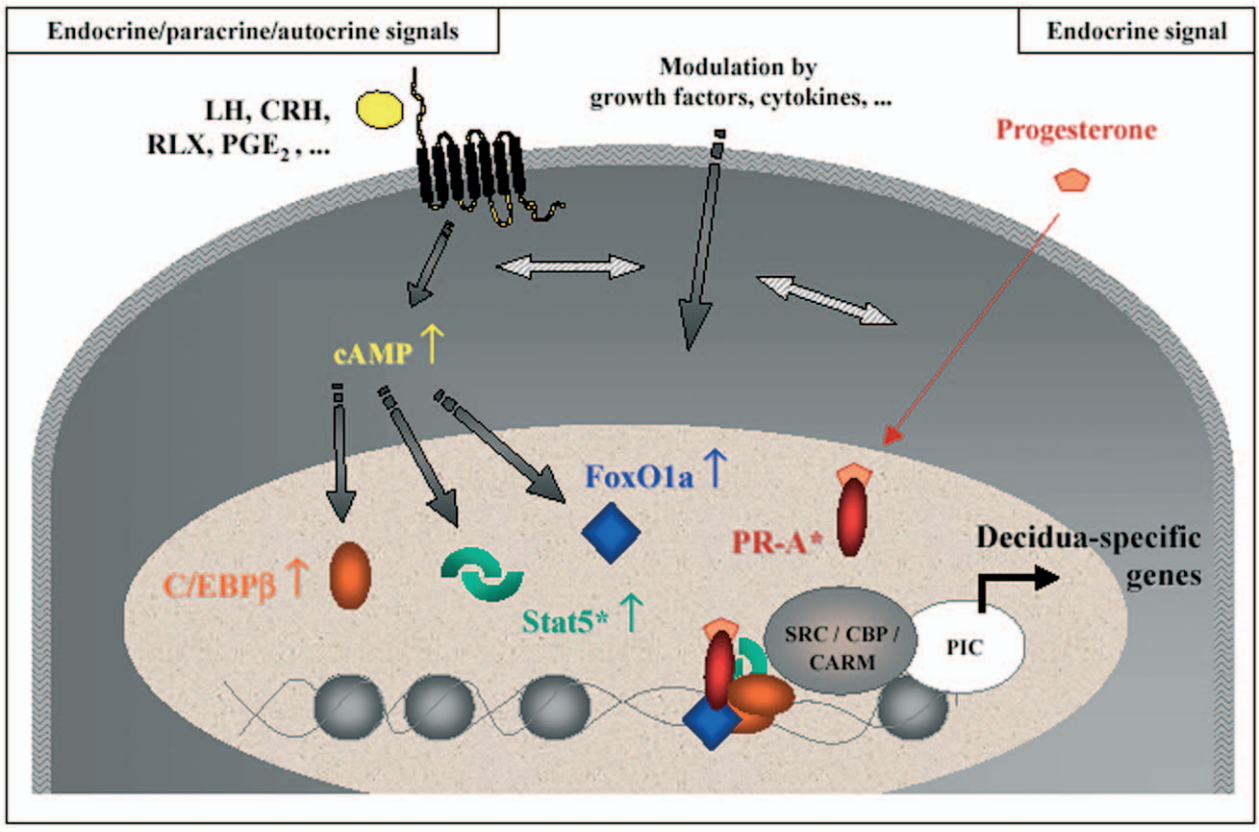

Figure 4 Model for cross-talk between PR-A and cAMP signalling in differentiating human ESC. Elevation of cAMP upon activation of GPCRs promotes expression and nuclear accumulation of C/EBPβ, FoxO1a and activated Stat5 (*). These factors can interact with ligand-activated PR-A (*) to initiate transcription of decidua-specific genes. For detailed explanation see text. PIC, preinitiation complex.

a ligand-dependent manner. Conversely, overexpression of LIP, but not LAP, enhances PR-B transactivation of single and complex progesterone response element-dependent promoters (Christian *et al.* 2002*a*). Recently, another bZIP transcription factor, Jun dimerization protein 2 (JDP-2), was identified capable of interacting with the DBD of PR, thereby stabilizing the interaction with general coactivators, and enhancing PR-B-dependent transcription (Wardell *et al.* 2002). JDP-2 belongs to the AP-1 family of transcription factors and, like LIP, lacks a transactivation domain and functions as a repressor of c-Jun transactivation of AP-1 response elements. In the context of decidualization, however, PR-A and LAP are the predominant isoforms and, hence, progesterone may be essential for maintaining and enhancing the expression of C/EBPβ-dependent genes in the decidua. Further support for the important role of PR-A in decidualization stems from the finding that the expression of IGFBP-1, another major decidual product which is regulated by progesterone and cAMP signalling (Tseng *et al.* 1992, Frank *et al.* 1994, Kim *et al.* 1998), is more strongly induced by ligand-activated PR-A than PR-B (Gao *et al.* 2000).

PR, FoxO1a and Stat5 In addition to C/EBPβ, FoxO1a and Stat5 are cAMP-induced transcription factors in decidualizing ESC and have also been shown to interact with PR (Richer *et al.* 1998, Schuur *et al.* 2001, Zhao *et al.* 2001, Christian *et al.* 2002*b*, Mak *et al.* 2002). Induction of the dPRL promoter by cAMP plus progestin is markedly enhanced by Stat5 and abolished by coexpression of a dominant negative to Stat5 (Mak *et al.* 2002). FoxO1a enhances the activity of the dPRL promoter cooperatively with C/EBPβ through the discrete −332/−270 region which also harbours the imperfect PR binding site (Christian *et al.* 2002*b*). Intriguingly, FoxO1a and C/EBPβ, in addition to PR, are also involved in transcriptional regulation of the IGFBP-1 promoter (Ghosh *et al.* 2001, Kim *et al.* 2003).

Convergence of progesterone and cAMP signalling

Taken together, these observations suggest a role for PR as a platform for the formation of a decidua-specific transcriptional complex involving such diverse transcription factors as FoxO, C/EBP and Stat5 (Fig. 4). This model is in

Table 2 Mutual enhancement of decidualization by progesterone and cAMP-mediated stimulation *in vitro*

		Enhancement of	Reference
Effector 1	**Effector 2**		
cAMP	Progesterone	PRL production dPRL promoter activity	Brosens *et al.* (1999)
CRH	Progesterone	PRL production	Ferrari *et al.* (1995)
hCG	Progesterone	PRL production	Nemansky *et al.* (1998)
PGE_2	Progesterone	cAMP production	Houserman *et al.* (1989)
Progesterone	RLX	PRL mRNA expression IGFBP-1 mRNA expression	Tseng *et al.* (1992)
Progesterone	PGE_2	PRL production IGFBP-1 production	Frank *et al.* (1994)

keeping with a number of additional observations. First, treatment of ESC with antiprogestins inhibits cAMP-induced dPRL expression which suggests that even the unliganded PR may be recruited in a ternary, albeit less functional, complex. Furthermore, in the absence of progesterone this transcriptosome is likely to be unstable as demonstrated by the inability of cAMP to maintain the expression of decidua-specific genes in long-term cultures (Fig. 2). Finally, transient and stable overexpression of either PR isoforms inhibit cAMP-induced dPRL promoter activity and protein expression respectively (Brosens *et al.* 1999). This could be explained by the ability of suprastochiometric PR levels to interfere with the assembly of a functional complex through squelching and sequestering of essential transcriptional partners and coactivators.

Intriguingly, microarray studies in the breast cancer cell line T47D identified STAT5, C/EBPβ, and FoxO1a as genes under direct transcriptional control of PR (Richer *et al.* 2002). Although the mechanism underlying these paradoxical cell-specific responses is not known, it may suggest that in the human endometrium PR only acquires full transcriptional activity in the presence of elevated cAMP levels. Steroid hormone receptors and their SRC complexes have been identified as down-stream targets of a variety of different signalling pathways, including PKA, PI3K/PKB, and MAPK (Lange *et al.* 1998, 2000, Rowan *et al.* 2000*a*,*b*, Campbell *et al.* 2001, Shen *et al.* 2001). Furthermore, the abundance and activity of many transcription factors, including PR, are regulated by diverse posttranslational modifications, including acetylation, ubiquitination, and sumoylation. It therefore appears possible that targeted covalent modification of PR and/or its coactivators, in response to cAMP-dependent activation of discrete or diverse signal transduction pathways, is necessary for full transcriptional competence.

Hormonal control of decidualization – from cultured ESC to the intact uterus

Numerous examples illustrate the mutual potentiation of cAMP- and progestin-stimulated effects on cultured ESC (Table 2). With the accumulated evidence for a convergence of cAMP- and PR-signalling pathways derived from *in vitro* models – what are the correlates *in vivo*? The key role of progesterone in orchestrating remodelling of the postovulatory endometrium is unquestioned. However, the input of GPCR-mediated signals *in vivo*, beyond the indirect influence of LH and follicle-stimulating hormone (FSH) acting on the ovary, may not have been fully acknowledged. The preovulatory LH surge, followed by the FSH peak, may provide an initiating signal by directly acting on endometrial gonadotrophin receptors. LH/hCG receptors, along with various G-protein subunits including Gsα, are present in membrane preparations from endometrial biopsies. In artificial cycles under hormone replacement therapy, Gsα was found to increase with the administration of progesterone for 3–9 days (Bernardini *et al.* 1995). Stimulatable adenylate cyclase activity in the human endometrium increases during the menstrual cycle, and the cAMP content in biopsies obtained from patients during the secretory phase is higher than that in the proliferative phase (Bergamini *et al.* 1985, Tanaka *et al.* 1993). As the secretory phase progresses, circulating LH and FSH levels fall, but paracrine/autocrine mechanisms such as the local production of RLX, CRH and hCG β-subunit may serve to sustain cAMP signalling (Wolkersdörfer *et al.* 1998, Gravanis *et al.* 1999, Palejawa *et al.* 2002). Furthermore, PGE synthase and its product, PGE_2, are found in the endometrium throughout the cycle (Milne *et al.* 2001). In case of successful implantation, the trophoblast-derived hCG may take over the gonadotrophic stimulation of the decidua. The notion that progesterone-dependent differentiation of the endometrium requires elevated intracellular cAMP levels is being exploited in clinical practice. For instance human menopausal gonadotrophins (hMG) and hCG have been used to treat women with unexplained recurrent miscarriages, although larger controlled trials are required to confirm the efficacy of this approach (Scott & Pattison 2000, Li *et al.* 2002).

The highly defined spatial and temporal pattern of differentiation of the human endometrium is likely to be controlled by a balance of inhibitory and stimulatory

signals. The endometrium is known to express several factors capable of inhibiting decidual PRL expression *in vitro*, including annexin-1, retinoic acid, transforming growth factor (TGF)-β, endothelins, and proinflammatory cytokines such as interleukin (IL)-1, tumour necrosis factor (TNF)-α, and interferon (IFN)-γ (Kariya *et al.* 1991, Pihoker *et al.* 1991, Chao *et al.* 1993, Jikihara & Handwerger 1994, Brar *et al.* 1996, Kubota *et al.* 1997, Christian *et al.* 2001*b*). Recently, we demonstrated that treatment with IFN-γ, resulting in activation of the Stat1 signalling pathway, potently represses dPRL promoter activity in differentiating ESC (Christian *et al.* 2001*b*). IFN-γ, or type II interferon, is an immunomodulatory T helper 1-type cytokine secreted predominantly by activated T lymphocytes and natural killer cells. In the endometrium, IFN-γ expression is markedly, albeit transiently, increased in the early secretory phase of the cycle. This peak of IFN-γ expression coincides with the influx of uterine NK cells but precedes differentiation of perivascular endometrial stromal cells (Kumar *et al.* 2001). An altered profile of uterine NK cells and persistently elevated endometrial IFN-γ expression in the secretory phase of the cycle has been documented in women with a history of recurrent miscarriages (Lachapelle *et al.* 1996, Lim *et al.* 2000). These observations underscore the notion that events in the conception cycle have a profound impact on subsequent pregnancy outcome.

Conclusion

The physiological importance of the decidual reaction in ensuring appropriate placenta formation and function is beyond doubt. Furthermore, from a clinical viewpoint, a spectrum of pregnancy disorders, including recurrent miscarriages, fetal growth restriction, placental abruption, and preeclampsia, are caused by uteroplacental dysfunction characterized by impaired decidual response, aberrant immune reaction, and spiral artery vasculopathy (Brosens *et al.* 2002). From a biochemical perspective, decidualization appears at first a simple affair, requiring only elevation of the intracellular cAMP levels to sensitize the cells to the actions of progesterone. However, scratch beyond the surface and a myriad of signalling pathways, transcription factors and cross-talks are required to coordinate this temporary reprogramming of the endometrial stroma. Our understanding of the molecular mechanisms of ESC differentiation is far from complete and there is a long way still to go before we can translate this knowledge into effective new treatments for common reproductive disorders.

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