

# An additive interaction between the NF $\kappa$ B and estrogen receptor signalling pathways in human endometrial epithelial cells

A.E. King<sup>1</sup>, F. Collins<sup>2</sup>, T. Klonisch<sup>3</sup>, J.-M. Sallenave<sup>4,5</sup>,  
H.O.D. Critchley<sup>1</sup>, and P.T.K. Saunders<sup>2,6</sup>

<sup>1</sup>Reproductive & Developmental Sciences, University of Edinburgh, Edinburgh, UK <sup>2</sup>Medical Research Council Human Reproductive Sciences Unit, Centre for Reproductive Biology, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, UK

<sup>3</sup>Department of Human Anatomy & Cell Science, University of Manitoba, Winnipeg, Canada <sup>4</sup>Universite Denis Diderot, Paris 7, France

<sup>5</sup>Unite de Defense Innee et Inflammation, INSERM U874, Batiment Metchnikoff, Institut Pasteur, 25, rue du Dr Roux, 75724 Paris Cedex, France

<sup>6</sup>Correspondence address. Tel: +44-131-242-9100; Fax: +44-131-242-6197; E-mail: p.saunders@hrsu.mrc.ac.uk

**BACKGROUND:** Human embryo implantation is regulated by estradiol (E2), progesterone and locally produced mediators including interleukin-1 $\beta$  (IL-1 $\beta$ ). Interactions between the estrogen receptor (ER) and NF kappa B (NF $\kappa$ B) signalling pathways have been reported in other systems but have not been detailed in human endometrium.

**METHODS AND RESULTS:** Real-time PCR showed that mRNA for the p65 and p105 NF $\kappa$ B subunits is maximally expressed in endometrium from the putative implantation window. Both subunits are localized in the endometrial epithelium throughout the menstrual cycle. Reporter assays for estrogen response element (ERE) activity were used to examine functional interactions between ER and NF $\kappa$ B in telomerase immortalized endometrial epithelial cells (*TERT*-EEC). E2 and IL-1 $\beta$  treatment of *TERT*-EECs enhances ERE activity by a NF $\kappa$ B and ER dependent mechanism; this effect could be mediated by ER $\alpha$  or ER $\beta$ . E2 and IL-1 $\beta$  also positively interact to increase endogenous gene expression of prostaglandin E synthase and c-myc. This is a gene-dependent action as there is no additive effect on cyclin D1 or progesterone receptor expression.

**CONCLUSION:** In summary, we have established that NF $\kappa$ B signalling proteins are expressed in normal endometrium and report that IL-1 $\beta$  can enhance the actions of E2 in a cell line derived from healthy endometrium. This mechanism may allow IL-1 $\beta$ , possibly from the developing embryo, to modulate the function of the endometrial epithelium to promote successful implantation, for example by regulating prostaglandin production. Aberrations in the interaction between the ER and NF $\kappa$ B signalling pathways may have a negative impact on implantation contributing to pathologies such as early pregnancy loss and infertility.

**Key words:** NF $\kappa$ B / estrogen receptor / endometrial epithelium / implantation

## Introduction

The human endometrium is a dynamic tissue that undergoes cycles of proliferation, differentiation, breakdown and repair (Jabbour *et al.*, 2006; van Mourik *et al.*, 2009). The development of the endometrium in preparation for implantation of the fertilized conceptus is under the control of the sex steroid hormones, estradiol and progesterone. Implantation occurs during the mid-late secretory phase of the menstrual cycle (the 'implantation window') and is characterized as an inflammatory event associated with increased expression of inflammatory mediators and immune cell infiltration (van Mourik *et al.*, 2009).

The actions of estrogens on the endometrium are mediated by estrogen receptors (ER) $\alpha$  and ER $\beta$  both of which are expressed in epithelial and stromal cells (Critchley *et al.*, 2001, 2002; Critchley and Saunders 2009). Immunoexpression of ER $\alpha$  varies during the normal cycle with intense staining in epithelial and stromal cells during the proliferative phase but a marked reduction in the functional layer during the late secretory phase (Lessey *et al.*, 1988; Critchley *et al.*, 2001). In contrast, ER $\beta$  alone is expressed in both endothelial and uterine natural killer cells (Henderson *et al.*, 2003) and appears similar in epithelial and stromal cells during both the proliferative and secretory phases (Critchley *et al.*, 2002). As a result ER $\beta$  appears to be the

predominant isoform in both the glandular (Critchley *et al.*, 2002) and surface (Bombail *et al.*, 2008) epithelium during the implantation window.

Inflammatory events including immune cell infiltration can be mediated via activation of NF kappa B (NFκB)-dependent gene transcription. NFκB is a dimeric transcription factor consisting of homo- or hetero-dimeric complexes of the Rel family of proteins; in the inactive state the complex is sequestered in the cytoplasm by a family of endogenous inhibitors, called IκBs. Previous studies have reported that expression of the NFκB subunits, p65 (Rel A) and p50, are increased during the mid secretory phase (Laird *et al.*, 2000; Page *et al.*, 2002) although expression of IκBα is reported to fall at this time (Page *et al.*, 2002). These semi-quantitative immunohistochemical studies would be consistent with a role for NFκB activation during the implantation window. However, this has not been confirmed using quantitative analysis.

Estrogens are well documented as having anti-inflammatory effects whereas activated NFκB initiates and maintains cellular inflammatory responses (Biswas *et al.*, 2005; Harnish, 2006). Evidence for the anti-inflammatory effects of estrogens was highlighted by the observation that pregnancy reduces the symptoms of rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease (Harnish, 2006). One mechanism by which estrogens can modulate inflammatory events has been described in articles documenting that reciprocal inhibitory cross-talk exists between NFκB and ER both *in vitro* (Harnish *et al.*, 2000; Feldman *et al.*, 2007; Quaedackers *et al.*, 2007) and *in vivo* (Evans *et al.*, 2001). For example, direct interactions between ERα and p65 (Quaedackers *et al.*, 2007), as well as cofactor involvement and modulation of IκBα expression by E2 (Nakshatri *et al.*, 1997; Sun *et al.*, 1998) have been reported. However, more recent studies have also reported that a positive interaction between NFκB and ER signalling can occur. For example, Adamson *et al.* (2008) found that treatment with E2 plus TNFα had a synergistic effect on estrogen response element (ERE)-dependent activation of the human prolactin gene in GH3 pituitary cells. Similarly, proinflammatory cytokines (TNFα, IL-1β or IL-6) and E2 also act synergistically to up-regulate expression of prostaglandin E synthase (PGES) mRNA levels in MCF7 breast cancer cells (Frasor *et al.*, 2008). As inflammation and steroid hormone action both play critical roles in endometrial function, we aimed to investigate whether cross-talk between NFκB and ER signalling occurs in endometrial epithelial cells and whether this is inhibitory or stimulatory in nature.

## Materials and Methods

### Patients and tissue collection

Endometrial samples ( $n = 24$ ) was collected from women undergoing procedures for benign gynaecological conditions. All women had regular menstrual cycles and had not undergone hormonal treatment in the 3 months preceding biopsy. Histological dating of the samples was performed according to the criteria of Noyes (1950). Serum samples collected at the time of endometrial biopsy were used for determination of circulating estradiol and progesterone concentrations by radioimmunoassay (Table I). These circulating steroid hormone levels were consistent with the histological assessment that was undertaken by an expert histologist. Tissues were either fixed in 4% neutral buffered formalin overnight at 4°C and embedded in paraffin wax according to standard procedures or placed

in RNA Later (Ambion/Applied Biosystems, Warrington, UK) for subsequent RNA extraction. Written informed consent was obtained from all patients and ethical approval was granted by the Lothian research ethics committee.

### Cell culture

Telomerase immortalized endometrial epithelial cells (*TERT*-EEC) were originally derived from normal endometrium obtained during the proliferative phase. These cells express ERα, ERβ and PR mRNA and protein (Hombach-Klonisch *et al.*, 2005; Collins, unpublished). Cells were seeded in 24 well culture plates (Corning-Costar) at a density of  $1 \times 10^5$ /well in Hams F-10 medium supplemented with 10% charcoal stripped fetal calf serum (Sigma, Dorset, UK), insulin–transferrin–selenium (ITS,  $1 \times$ ; Lonza, Slough, UK), penicillin/streptomycin and gentamycin (Sigma). Ishikawa cells (ECACC, Salisbury, UK) were originally derived from the endometrial adenocarcinoma from a 39-year-old woman (Nishida *et al.*, 1985). In our hands, this line of Ishikawa cells expresses ERα, ERβ and PR; cells were seeded in 24 well culture plates at a density of  $1 \times 10^5$ /well in DMEM medium supplemented with 10% charcoal stripped fetal calf serum, L-glutamine, penicillin/streptomycin and non-essential amino acids (1%).

### Luciferase reporter assays

An adenoviral vector containing a 3xERE-tk-luciferase reporter gene was prepared by subcloning a piece of DNA containing the ERE promoter and luciferase sequences from a plasmid that was a kind gift from Professor DP McDonnell [(Hall and McDonnell, 1999), Duke University NC, USA] into the adenoviral genome (pBHGloxΔE1.3Cre, Microbix) that was propagated in Hek293 cells according to standard methods. An adenovirus containing an NFκB response element linked to a luciferase reporter gene was obtained from Vector Biolabs (Philadelphia, USA). The control adenovirus (Ad-d1703) and an adenovirus containing a dominant negative IκBα mutant (Ad-IκBα) have been described previously (Jobin *et al.*, 1997; Henriksen *et al.*, 2004). Luciferase was detected using the Bright-Glo luciferase assay system (Promega, Southampton, UK) as detailed in the manufacturer's instructions.

Three separate experimental protocols were used to examine the effects of E2 and IL-1β on ERE activity in *TERT*-EEC. In all experiments, cells were infected with adenovirus containing the ERE-luc expression construct. The total multiplicity of infection (MOI) was 100.

- (i) Cells were left untreated or treated with the ER antagonist, ICI 182720 ( $10^{-6}$  M), for 1 h. Thereafter cells were either left untreated or incubated with vehicle (DMSO), E2 ( $10^{-8}$  M), IL-1β (0.1 ng/ml) or E2 + IL-1β for a further 24 h.
- (ii) Cells were co-infected with a control adenovirus (Ad-d1703) or an adenovirus containing a dominant negative IκBα mutant (Ad-IκBα) at a total MOI of 100. The following day, cells were either left untreated or treated with vehicle (DMSO), E2 ( $10^{-8}$  M; Sigma), IL-1β (0.1 ng/ml; Peprotech, London, UK) or E2 + IL-1β and incubated for a further 24 h.
- (iii) Cells were co-infected with a control adenovirus (Ad-d1703) or an adenovirus containing a dominant negative IκBα mutant (Ad-IκBα) at a total MOI of 100. Cells were either left untreated or treated with vehicle (DMSO), IL-1β (0.1 ng/ml), the ERα selective agonist, PPT (4,4',4''-(4-propyl-[<sup>1</sup>H]-pyrazole-1,3,5-triyl tris-phenol (Kraichely *et al.*, 2000)) at  $10^{-8}$  M, the ERβ selective agonist, DPN (2,3-bis(4-hydroxyphenyl)-propionitrile (Meyers *et al.*, 2001)) at  $10^{-7}$  M, or combinations of these treatments (PPT + IL-1β or DPN + IL-1β) and incubated for 24 h.

**Table 1** Details of endometrial biopsies

Cycle phase	Number of samples	E2 (pM), range median	P4 (nM), range median
PROL	7	78.91–559.09, 311	0.97–5.91, 2.47
ES	5	289–599.12, 504.3	5.5–112.91, 80.5
MS	6	279–1949, 659.2	42.10–114.53, 78.6
LS	4	59.09–819, 172.1	1.06–11.29, 5.4
MENST	2	371, 223	7.54, 2.98

Cycle phase (as determined by the Noyes' criteria) was consistent with circulating estradiol and progesterone concentrations at time of biopsy. PROL = proliferative; ES, MS and LS = early, mid and late secretory; MENST = menstrual.

Concentrations of E2, PPT and DPN used were those found to give an optimal response in the ERE reporter assay in pilot studies. In additional complementary experiments, Ishikawa cells were infected with ERE and either Ad-d1703 or Ad-I $\kappa$ B $\alpha$  (total MOI = 50) and treated as in (ii).

The impact of treatments on NF $\kappa$ B signalling was investigated by infecting TERT-EECs with the NF $\kappa$ B-luc reporter at an MOI of 50; cells were treated with vehicle, E2 ( $10^{-8}$  M), IL-1 $\beta$  (0.1 ng/ml) or E2 + IL-1 $\beta$  for 24 h. Each experiment was repeated on three separate occasions.

## Endogenous gene expression

TERT-EEC were seeded in six well culture plates at a density of  $3 \times 10^5$ /well in Hams-F10 medium supplemented with 10% charcoal stripped fetal calf serum, ITS (1 $\times$ ), penicillin/streptomycin and gentamycin. The following day cells were either left untreated or incubated with vehicle (DMSO), E2 ( $10^{-8}$  M), IL-1 $\beta$  (0.1ng/ml) or both factors at the same time for 2, 4 and 8 h. The experiment was repeated three times.

## Quantitative RT-PCR

RNA was extracted from cells or endometrial tissues using RNeasy mini kits (Qiagen); RNA samples were treated with DNase I. Complementary DNA was prepared from 400 ng of template RNA in 20  $\mu$ l reactions containing: RT buffer (1 $\times$ ), magnesium chloride (5.5 mM), dNTP mix (2 mM), random hexamers (2.5  $\mu$ M), RNase inhibitor (0.4 U/ $\mu$ l) and Multiscribe reverse transcriptase (1.25 U/ $\mu$ l; Applied Biosystems, Cheshire, UK). Each cDNA preparation included two controls: one containing template RNA but no reverse transcriptase (RT negative) and the other containing reverse transcriptase with water in place of template RNA (RT H<sub>2</sub>O).

PCR reaction mixtures contained Taqman Master-mix (1 $\times$ ; Applied Biosystems), forward and reverse primers (Eurogentec) and probe (Eurogentec/Roche) for the gene of interest and forward and reverse primers and probe for ribosomal 18s (Applied Biosystems). Expression of the gene of interest was related to expression of 18S ribosomal RNA and to an internal control sample using the  $2^{-\Delta\Delta C_t}$  method. Controls included the RT controls detailed above and a PCR no template control (water in place of cDNA). PCR reactions were run on an ABI 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems, USA). Primer and probe sequences are detailed in Supplementary data, Table S1.

## Immunohistochemistry

Immunohistochemical localization of p65, p105/p50 and I $\kappa$ B $\alpha$  was performed on endometrial ( $n = 17$ ) sections as follows. Antigen retrieval was carried out using a microwave (15 min in antigen unmasking solution, Vector, Peterborough, UK); endogenous peroxidase activity was blocked with 3% hydrogen peroxide (Sigma-Aldrich, Dorset, UK). Additional pre-treatments involved incubation with solutions from the avidin biotin blocking

kit (Vector) and the DakoCytomation protein block (Dako, Ely, UK), 10 min each at room temperature. Sections were incubated overnight at 4°C with either rabbit-anti p65 (1:500; Santa Cruz), rabbit anti-p105/50 (1:500; NLS, Santa Cruz) or rabbit anti-I $\kappa$ B $\alpha$  (1:300; E130, Abcam, Cambridge, UK) diluted in REAL antibody diluent (Dako). For negative controls, the primary antibody was substituted with antibody diluent alone. Sections were washed and incubated with a biotinylated goat anti-rabbit secondary antibody and the avidin biotin peroxidase detection system, both for 30 min at room temperature (Vectastain Elite ABC, Vector). Positive staining was detected using diaminobenzidine (ImmPACT DAB; Vector) and sections were counterstained with Harris' haematoxylin.

## Statistics

Significant differences in mRNA expression in endometrial biopsies was determined by one-way ANOVA and Tukey's *post hoc* analysis. These data were logarithmically transformed prior to statistical analysis. Data from reporter assays were statistically analysed using repeated measures two-way ANOVA and Bonferroni's *post hoc* analysis. Vehicle treatments are not shown in figures as there was no statistical difference between vehicle and control (without vehicle) samples in any of the experiments. Fold changes quoted in the results section were calculated by comparison to the untreated control for IL-1 $\beta$  and by comparison to DMSO (vehicle control) for E2 and E2 + IL-1 $\beta$ . Significant differences in mRNA expression in cell culture experiments were determined using repeated measures two-way ANOVA and Bonferroni's *post hoc* analysis.

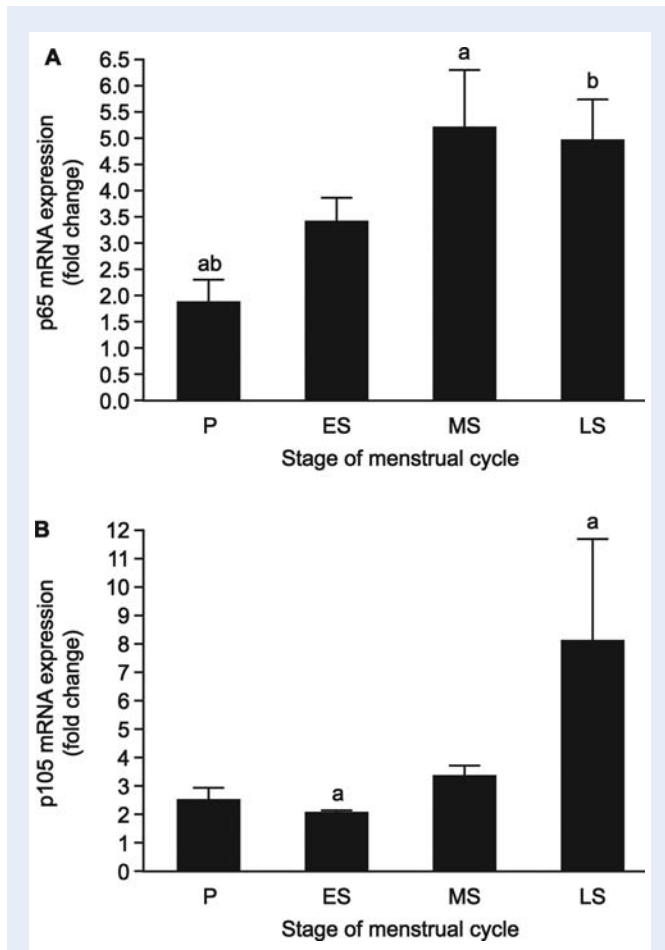
## Results

### Expression of p65 and p105 mRNA in endometrium is highest during the secretory phase of the menstrual cycle

Quantitative RT-PCR analysis of well characterized endometrial biopsies showed that p65 mRNA expression is highest during the mid and late secretory phases (Fig. 1A;  $P < 0.05$ ). p105 mRNA expression peaks during the late secretory phase of the menstrual cycle (Fig. 1B;  $P < 0.05$ ).

### p65, p105/p50 and I $\kappa$ B $\alpha$ are widely expressed in the human endometrium and are present in both epithelial and stromal compartments

Immunoeexpression of p65, p105/p50 and I $\kappa$ B $\alpha$  was detected in endometrium at all stages of the menstrual cycle (Fig. 2: shows immunolocalization in a representative endometrial biopsy from the mid



**Figure 1** Differential mRNA expression of p65 and p105 in endometrium from throughout the menstrual cycle.

P = proliferative; ES = early secretory; MS = mid secretory; LS = late secretory. Same letters denote statistical significance. (A) p65. p65 mRNA expression is maximal during the mid and late secretory phase of the menstrual cycle. ab:  $P < 0.05$  (B) p105. p105 mRNA expression peaks in the late secretory of the menstrual cycle. a:  $P < 0.05$ .

secretory phase). There were no obvious changes to the pattern of localization at different menstrual cycle phases (data not shown). Cytoplasmic staining was detected in both glandular and stromal compartments as well as in endothelial cells surrounding the blood vessels.

### IL-1 $\beta$ and E2 interact to enhance ERE-dependent reporter gene activity in endometrial epithelial cells and this is ER dependent

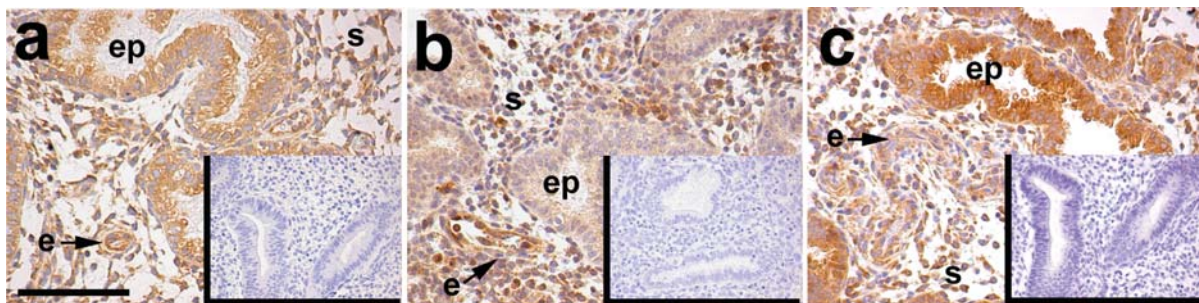
Initial studies conducted using endometrial adenocarcinoma Ishikawa cells showed that treatment with E2 raised ERE activity 4-fold whereas addition of IL-1 $\beta$  alone or in combination with E2 had no impact on reporter gene activity (Supplementary data, Fig. S1;  $P < 0.01$ ).

Incubation of *TERT*-EECs with  $10^{-8}$  M E2 resulted in a 5-fold increase in the ERE-dependent expression of luciferase (Fig. 3). In contrast with the Ishikawa cells, increased expression was also observed when cells were treated with IL-1 $\beta$  alone and simultaneous exposure to E2 and IL-1 $\beta$  increased ERE-luciferase reporter activity 15-fold (Fig. 3;  $P < 0.01$ ). Both basal ERE activity and the increased ERE activity that occurred when *TERT*-EEC were treated with E2, IL-1 $\beta$  or E2 + IL-1 $\beta$  were abolished by the presence of the ER antagonist, ICI 182720.

In complementary studies, NFκB response element activity was increased when *TERT*-EEC were incubated with IL-1 $\beta$  alone. However, incubation with E2 had no impact on expression of the luciferase reporter and there was no evidence of an additive or synergistic effect when cells were co-incubated with E2 and IL-1 $\beta$  (data not shown).

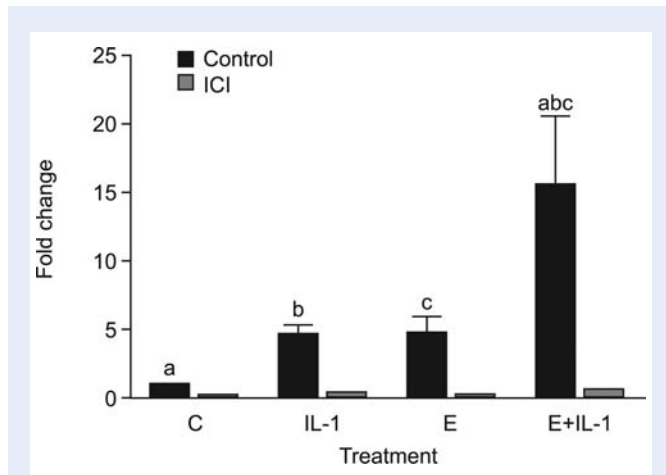
### Activation of ERE-dependent reporter gene activity by IL-1 $\beta$ is NFκB-dependent and the interaction between NFκB and ER signalling pathways may be mediated by either ER $\alpha$ or ER $\beta$

In a similar experiment assessing ERE activation, the presence of a dominant negative IκB $\alpha$  molecule reduced IL-1 $\beta$ -dependent increases



**Figure 2** Immunolocalization of p65, p105/p50 and IκB $\alpha$  in endometrium from the mid secretory phase of the menstrual cycle.

There were no obvious changes in the pattern of localization in endometrial biopsies from across the menstrual cycle. The pattern of localization is demonstrated in a representative endometrial biopsy from the mid secretory phase. (A) p65. (B) p50. (C) IκB $\alpha$ . Negative controls in insets. Scale bar = 100  $\mu$ m. ep = epithelium; s = stroma; e = endothelium.



**Figure 3** E2 and IL-1 $\beta$  positively interact to enhance estrogen response element (ERE) activity in an estrogen receptor (ER) dependent manner in *TERT*-EECs.

Cells were treated with E2, IL-1 $\beta$  and E2 + IL-1 $\beta$  in the absence and presence of the ER antagonist, ICI 172820 ( $n = 3$ ). Same letters denote statistical significance. a:  $P < 0.001$ ; bc:  $P < 0.01$ .

in ERE-luciferase gene expression when cells were incubated with IL-1 $\beta$  alone or IL-1 $\beta$  plus E2 (Fig. 4A).

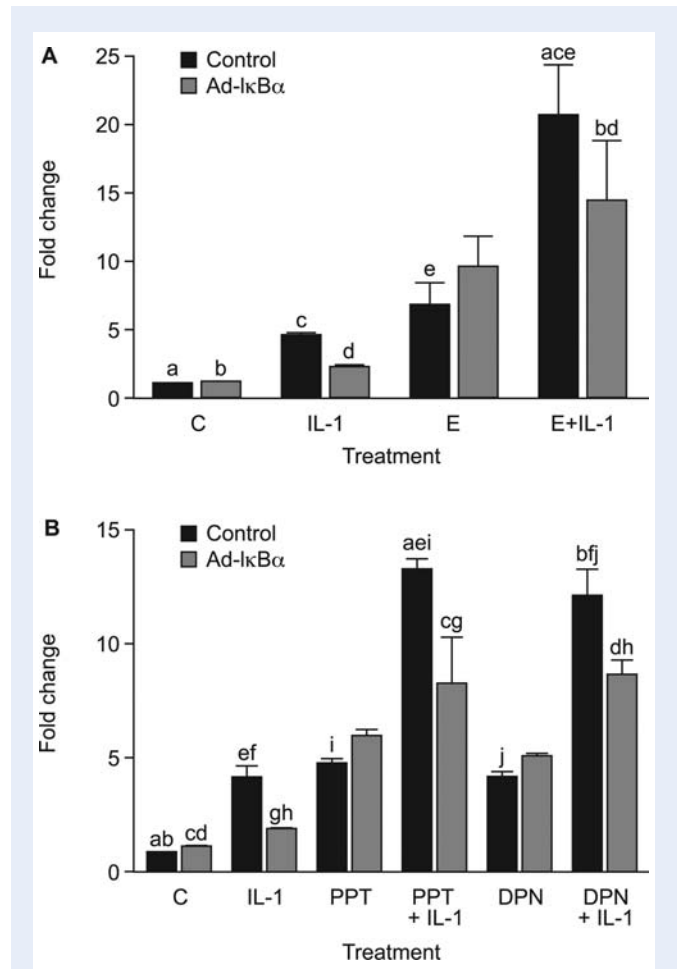
Treatment of *TERT*-EEC with the ER $\alpha$  selective agonist, PPT, or the ER $\beta$  selective agonist, DPN, increased ERE-luciferase reporter gene expression 5-fold and 4-fold, respectively. When the cells were treated with IL-1 $\beta$  in combination with PPT or DPN, ERE activity was raised 13-fold and 12-fold, respectively (Fig. 4B;  $P < 0.001$ ), and these additive interactions were reduced by the presence of Ad-I $\kappa$ B $\alpha$ .

### IL-1 $\beta$ and E2 act additively to enhance expression of PGES and c-myc

Expression of PR<sub>A+B</sub>, c-myc, PGES and cyclin D1 in *TERT*-EEC was examined after 2, 4 and 8 h of treatment. PR<sub>A+B</sub> mRNA expression was significantly increased by treatment with E2 at 4 and 8 h and this was unaffected by treatment with IL-1 $\beta$  (Fig. 5A;  $P < 0.05$ ). c-myc mRNA expression was up-regulated 3.4-fold by treatment with E2 alone at 8 h and this increase was enhanced in the presence of IL-1 $\beta$  (6.7-fold increase, Fig. 5B;  $P < 0.05$ ). Treatment with IL-1 $\beta$  alone did not alter c-myc mRNA expression. Expression of PGES mRNA in *TERT*-EEC was increased 2.7-fold and 4.8-fold by treatment with E2 and IL-1 $\beta$  for 8 h, respectively. E2 and IL-1 $\beta$  acted together to raise PGES mRNA expression by 9.5-fold (Fig. 5C;  $P < 0.001$ ). Similar effects of treatment were observed at 4 h. In contrast, cyclin D1 mRNA expression was increased 1.6-fold by treatment with E2 at 8 h and this response was not altered by the presence of IL-1 $\beta$  (Fig. 5D;  $P < 0.01$ ).

## Discussion

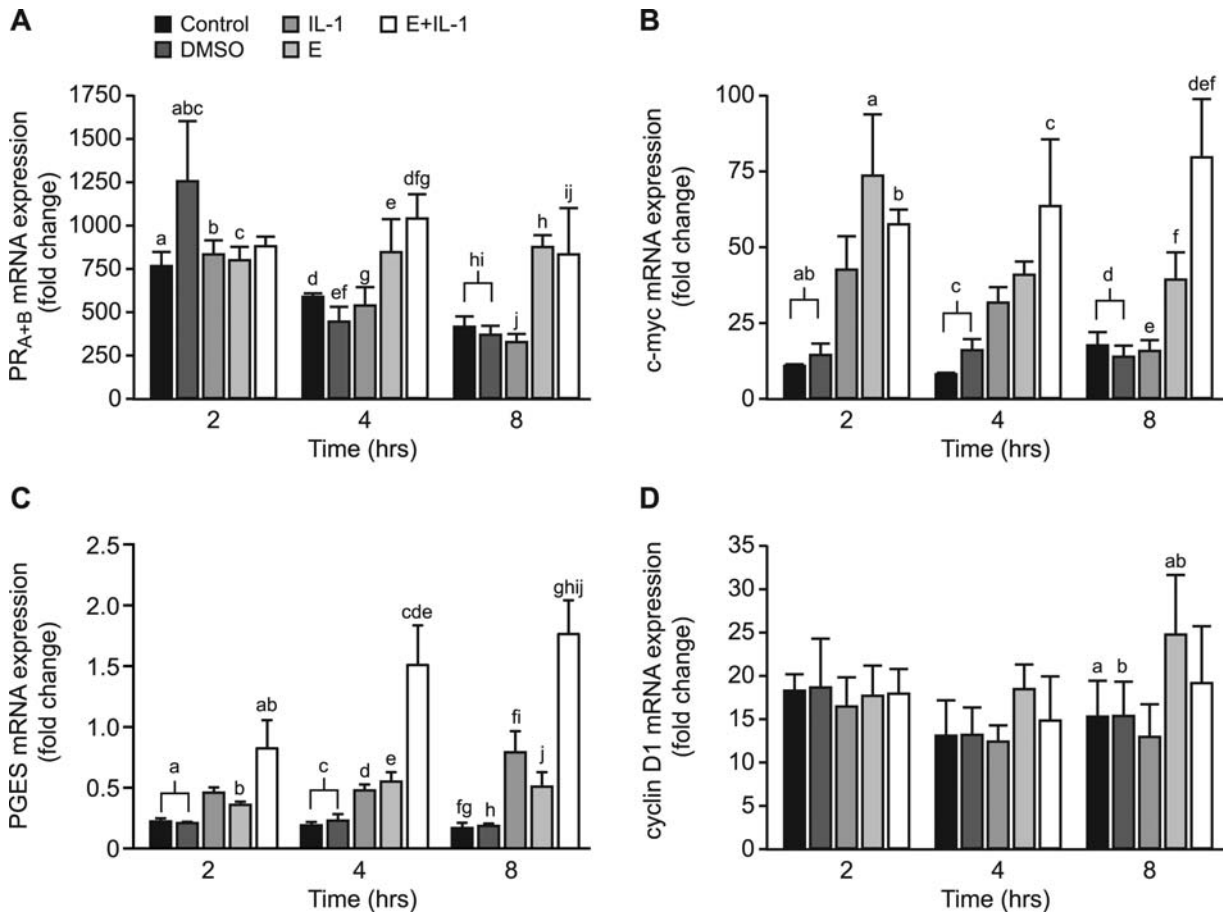
To our knowledge, we report novel data detailing an additive interaction between the ER and NF $\kappa$ B signalling pathways in endometrial epithelial cells derived from normal healthy endometrium (Hombach-Klonisch et al., 2005). The increase in ERE activity could be mediated by either ER $\alpha$  or ER $\beta$ .



**Figure 4** The positive interaction between E2 and IL-1 $\beta$  in *TERT*-EECs is NF $\kappa$ B dependent and can be mediated by ER $\alpha$  and ER $\beta$ .

Same letters denote statistical significance. (A) Cells were treated with E2, IL-1 $\beta$  and E2 + IL-1 $\beta$  in the absence and presence of an inhibitor of the NF $\kappa$ B pathway (Ad-I $\kappa$ B $\alpha$ ;  $n = 3$ ). ac:  $P < 0.001$ ; bde:  $P < 0.01$ . (B) Cells were treated with PPT, DPN, IL-1 $\beta$ , PPT + IL-1 $\beta$  and DPN + IL-1 $\beta$  in the absence and presence of Ad-I $\kappa$ B $\alpha$  ( $n = 3$ ). abefij:  $P < 0.001$ ; dh:  $P < 0.01$ ; cg:  $P < 0.05$ .

Previous studies have reported negative interactions between the ER and NF $\kappa$ B pathways. Mutual repression of signalling between NF $\kappa$ B and ER has been reported in various *in-vitro* systems (Harnish et al., 2000; Feldman et al., 2007; Quaedackers et al., 2007). A number of different mechanisms including direct interactions between p65 and ER (Quaedackers et al., 2007), cofactor recruitment (Harnish et al., 2000; Speir et al., 2000) and E2-mediated modulation of I $\kappa$ B $\alpha$  levels (Sun et al., 1998) have been suggested to explain these results. Direct interactions between ER $\alpha$  and p65 have been shown in osteoblastic U2-OS cells (Quaedackers et al., 2007) although E2 prevents degradation of I $\kappa$ B $\alpha$  by PMA treatment of HeLa cells (Sun et al., 1998). The cofactors CBP and p300 are reported to mediate interactions between NF $\kappa$ B and ER in human liver and coronary smooth muscle cells, respectively (Harnish et al., 2000; Speir et al., 2000). Mutual inhibition of NF $\kappa$ B and ER signalling has also been detailed in an *in-vivo* mouse model of cardiovascular disease (Evans et al., 2001). In a reproductive context, Feldman et al. (2007) have shown



**Figure 5** Regulation of endogenous gene expression by E2 and IL-1 $\beta$  in *TERT*-EECs.

Cells were treated with E2, IL-1 $\beta$  and E2 + IL-1 $\beta$  ( $n = 3$ ). Same letters denote statistical significance. (A) PR<sub>A+B</sub>. abcdehi:  $P < 0.05$ ; fgj:  $P < 0.01$  (B) c-myc. ade:  $P < 0.001$ ; c:  $P < 0.01$ ; bf:  $P < 0.05$ . (C) PGES. af:  $P < 0.01$ ; b:  $P < 0.05$ ; cdeghij:  $P < 0.001$ ;. (D) Cyclin D1. ab:  $P < 0.01$ .

that IL-1 $\beta$  inhibits ERE activity in Ishikawa cells in the presence of E2 after a 48 h treatment period and have suggested that there is also a reciprocal inhibitory action of E2 on NF $\kappa$ B activity. In addition, it has also been reported that NF $\kappa$ B activation reduces the response of both Ishikawa and endometrial stromal cells to E2 (Guzeloglu-Kayisli *et al.*, 2008). In our current study, we did not find any effect of IL-1 $\beta$  on ERE activity in Ishikawa cells in the absence or presence of E2 at a 24 h time point. These results are in contrast to the additive interaction that occurs between the ER and NF $\kappa$ B pathways in *TERT*-EECs, suggesting that the relationship between these two signalling pathways is cell-type specific. This may be reflective of differences between cells derived from the healthy endometrial epithelium and those derived from adenocarcinomas or the stromal compartment and highlights the importance of using untransformed cells to model normal endometrial cell function.

Positive interactions between the ER and NF $\kappa$ B signalling pathways have been described in some cell lines. For example, Frasor *et al.* (2008) reported that E2 and TNF $\alpha$  or IL-1 $\beta$  act in synergy to up-regulate PGES expression in the MCF-7 breast cancer cell line by an ER- and NF $\kappa$ B-dependent mechanism, which involves increased recruitment of both ER $\alpha$  and p65 to the ERE in the PGES promoter. In the present study, we demonstrated that expression of PGES was

markedly up-regulated when *TERT*-EEC were incubated with E2 and IL-1 $\beta$ , suggesting that p65 recruitment to the ERE may also occur in our *in-vitro* model. Alternatively interactions between NF $\kappa$ B and ER may be mediated via one of the mechanisms that have been documented to occur when there is a negative interaction between these signalling pathways (as detailed above). Further studies are necessary to determine the molecular basis for the interaction between the ER and NF $\kappa$ B pathways in *TERT*-EECs. The human prolactin promoter contains a functional ERE and is synergistically activated by E2 and TNF $\alpha$  in a reporter assay via an ER-dependent mechanism (Adamson *et al.*, 2008). Endogenous prolactin gene expression was also increased synergistically by E2 and TNF $\alpha$  in that study. However, a reporter assay using a consensus ERE-construct showed that TNF $\alpha$  inhibited ERE activity in this case suggesting that the synergistic interaction between TNF $\alpha$  and ER is specific to the prolactin promoter (Adamson *et al.*, 2008).

We found evidence of an additive interaction between E2 and IL-1 $\beta$  regulated expression of PGES and c-myc, but the same treatment did not have a similar impact on expression of PR or cyclin D1 in *TERT*-EEC. We suggest that these differences are due to the presence of different response elements in the promoter region of each gene. In this context, previous studies have suggested that promoter elements

within each of these genes can be regulated by NF $\kappa$ B (Duyao et al., 1990a, b; Guttridge et al., 1999; Takebayashi et al., 2003; Saitoh et al., 2005; Condon et al., 2006; Ackerman et al., 2008) but that their regulation by E2 is more complex. Notably ligand-activated ERs can regulate gene expression directly via an ERE or indirectly by interacting with other transcription factors including AP-1 and Sp1 (Gruber et al., 2004). ER-dependent regulation of PR is reported to be mediated by both EREs and a Sp1 site (Chauchereau et al., 1991; Petz et al., 2004). Although c-myc is considered to be an E2-regulated early response gene, its promoter does not contain a consensus ERE but its E2 responsiveness is reported to be mediated via Sp1 binding (Dubik and Shiu, 1992). The PGES promoter is reported to contain a functional ERE upstream of the transcription start site (Frasor et al., 2008) although the responsiveness of cyclin D1 to E2 is reported to involve AP-1, Sp1 and NF $\kappa$ B sites rather than a consensus ERE (Castro-Rivera et al., 2001; Rubio et al., 2006).

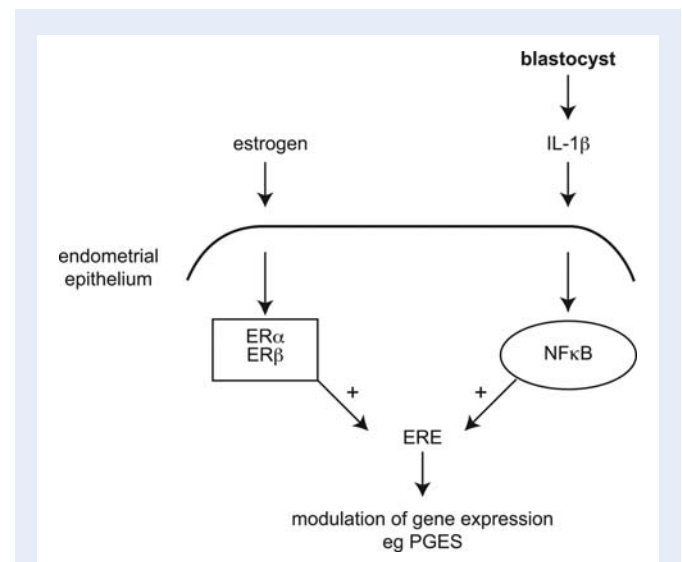
A positive interaction between the ER- and NF $\kappa$ B-signalling pathways has also been reported to occur at  $\kappa$ B sites. In the T47D breast cancer cell line, E2 induces proliferation and cyclin D1 expression by an NF $\kappa$ B dependent mechanism, which is enhanced in the presence of TNF $\alpha$  and involves the formation of a protein complex containing ER, p65 and the coactivator RAC3 (Rubio et al., 2006). In COS-1 cells, ER $\alpha$  and NF $\kappa$ B have also been found to have a synergistic action at the 5-HT1A receptor promoter via NF $\kappa$ B sites, although this study also showed that E2 represses the activity of an NF $\kappa$ B response element in a synthetic construct, again suggesting that the interaction between the pathways is promoter specific (Wissink et al., 2001). In contrast, in our current study, we have not found any evidence for a positive or negative effect of E2 on NF $\kappa$ B reporter activity in TERT-EECs (data not shown).

During the putative implantation window, immunoexpression studies have revealed that ER $\beta$  is the predominant ER protein present in the nuclei of glandular and surface epithelial cells in the functional layer of the endometrium (Critchley et al., 2002; Bombail et al., 2008). In the present study, we detected immunoexpression of the NF $\kappa$ B proteins, p65 and p50, in the glandular epithelium in endometrium along with the inhibitor protein, I $\kappa$ B $\alpha$ . Our previous studies have also demonstrated the presence of mRNA for the upstream kinases MEKK1, NIK, IKK $\alpha$  and IKK $\beta$  in endometrium with MEKK1 and IKK $\alpha$  proteins localized to the glandular and, in some cases luminal, epithelium (King et al., 2001). The expression of p65 and p50 has previously been reported to peak around the putative implantation window (Laird et al., 2000; Page et al., 2002) on the basis of semi-quantitative immunohistochemical studies. Our current quantitative analysis of p65 and p105 mRNA expression in well characterized endometrial biopsies is in agreement. It should be noted that these quantitative PCR studies do not provide information regarding which cellular compartment(s) are responsible for the increased expression of p65 and p105 mRNA in the secretory phase. Leukocyte populations present in endometrium vary across the menstrual cycle (Bulmer et al., 1991) and may contribute to the changes we have documented. In any case, our current data, along with that previously reported by others, suggest that the NF $\kappa$ B pathway may be involved in the regulation of inflammatory events at implantation.

The IL-1 system is a key cytokine network that has an impact on endometrial cell function at the time of implantation (Simon et al., 1997; Fazleabas et al., 2004). Notably, IL-1 is produced by the

preimplantation embryo (Baranao et al., 1997) and the IL-1 receptor type I is present in endometrial epithelium (Bigonnesse et al., 2001). An IL-1 receptor antagonist has been shown to prevent implantation in mice (Simon et al., 1994) although a further study did not report a similar effect (Abbondanzo et al., 1996). Our data suggest that IL-1 may augment the actions of E2 on the endometrial epithelium *in vivo*. This could be a mechanism to allow the developing blastocyst to modulate the function of the endometrial epithelium enhancing the likelihood of successful implantation (Fig. 6). For example, both cyclo-oxygenase and PGES enzymes are present in the human endometrial epithelium during the implantation window (Jones et al., 1997; Marions and Danielsson, 1999; Milne et al., 2001). Prostaglandins increase vascular permeability, modulate decidualization and regulate the immune response at implantation (Tranguch et al., 2005; Kennedy et al., 2007). Our data suggest that E2 and IL-1 $\beta$  may act additively to increase the production of PGE $_2$  by up-regulating PGES expression in the endometrial epithelium. We recognize that progesterone may also impact the ER and NF $\kappa$ B signalling pathways at implantation. However, the endometrial epithelium does not express the PR during the implantation window, so progesterone actions would be mediated via the stromal compartment (Wang et al., 1998). Although the TERT-EECs do express the PR, examination of progesterone effects in our *in-vitro* epithelial cell model would not be representative of the environment *in vivo*.

In summary, we have identified a novel positive interaction between the ER- and NF $\kappa$ B-dependent signalling pathways in an endometrial epithelial cell line derived from healthy endometrium. NF $\kappa$ B activation modulates ERE activity by an ER and NF $\kappa$ B dependent mechanism and can enhance ER-mediated endogenous gene expression in an *in-vitro* human endometrial epithelial cell model. The positive interaction



**Figure 6** Summary of the potential interactions between the ER and NF $\kappa$ B signalling pathways in endometrial epithelial cells.

E2 and IL-1 $\beta$  positively interact to enhance ERE activity in the endometrial epithelium and to up-regulate expression of a subset of genes, including PGES. This mechanism may allow the developing blastocyst, which produces IL-1, to modulate gene expression in the endometrial epithelium, enhancing the likelihood of successful implantation.

between NFκB and ER may be involved in the regulation of epithelial cell function at implantation and aberrant interactions between these signalling pathways may be involved in pathologies such as early pregnancy failure and infertility.

## Supplementary data

Supplementary data are available at <http://humrep.oxfordjournals.org/>.

## Authors' Roles

A.E.K., F.C.: laboratory work, data analysis, manuscript preparation. T.K., J.-M.S.: manuscript preparation. H.O.D.C.: data analysis, manuscript preparation. P.T.K.S.: study design, data analysis, manuscript preparation.

## Acknowledgements

We thank our research nurses Catherine Murray, Catherine Cairns and Sharon MacPherson for patient recruitment and assistance with biopsy collection, Pamela Brown for preparation of viral constructs, Amirah Mohamed for technical assistance and Ronnie Grant for graphical assistance.

## Funding

This research was supported by the Caledonian Research Foundation (personal research fellowship to Dr Anne King), the Barbour Watson Fund and Tenovus, Scotland. PTKS and FC were supported by MRC programme U.1276.00.002.00005.01. HODC was supported by MRC programme grant G0500047.

## References

- Abbondanzo SJ, Cullinan EB, McIntyre K, Labow MA, Stewart CL. Reproduction in mice lacking a functional type I IL-1 receptor. *Endocrinology* 1996;**137**:3598–3601.
- Ackerman WEt, Summerfield TL, Vandre DD, Robinson JM, Kniss DA. Nuclear factor-kappa B regulates inducible prostaglandin E synthase expression in human amnion mesenchymal cells. *Biol Reprod* 2008;**78**:68–76.
- Adamson AD, Friedrichsen S, Semprini S, Harper CV, Mullins JJ, White MR, Davis JR. Human prolactin gene promoter regulation by estrogen: convergence with tumor necrosis factor-alpha signaling. *Endocrinology* 2008;**149**:687–694.
- Baranao RI, Piazza A, Rumi LS, Polak de Fried E. Determination of IL-1 and IL-6 levels in human embryo culture-conditioned media. *Am J Reprod Immunol* 1997;**37**:191–194.
- Bigonnesse F, Labelle Y, Akoum A. Triphasic expression of interleukin-1 receptor type I in human endometrium throughout the menstrual cycle of fertile women and women with unexplained infertility. *Fertil Steril* 2001;**75**:79–87.
- Biswas DK, Singh S, Shi Q, Pardee AB, Iglehart JD. Crossroads of estrogen receptor and NF-kappaB signaling. *Sci STKE* 2005;**2005**:pe27.
- Bombail V, MacPherson S, Critchley HO, Saunders PT. Estrogen receptor related beta is expressed in human endometrium throughout the normal menstrual cycle. *Hum Reprod* 2008;**23**:2782–2790.
- Bulmer JN, Longfellow M, Ritson A. Leukocytes and resident blood cells in endometrium. *Ann N Y Acad Sci* 1991;**622**:57–68.
- Castro-Rivera E, Samudio I, Safe S. Estrogen regulation of cyclin D1 gene expression in ZR-75 breast cancer cells involves multiple enhancer elements. *J Biol Chem* 2001;**276**:30853–30861.
- Chauchereau A, Loosfelt H, Milgrom E. Phosphorylation of transfected wild type and mutated progesterone receptors. *J Biol Chem* 1991;**266**:18280–18286.
- Condon JC, Hardy DB, Kovaric K, Mendelson CR. Up-regulation of the progesterone receptor (PR)-C isoform in laboring myometrium by activation of nuclear factor-kappaB may contribute to the onset of labor through inhibition of PR function. *Mol Endocrinol* 2006;**20**:764–775.
- Critchley HO, Saunders PT. Hormone receptor dynamics in a receptive human endometrium. *Reprod Sci* 2009;**16**:191–199.
- Critchley HO, Brenner RM, Henderson TA, Williams K, Nayak NR, Slayden OD, Millar MR, Saunders PT. Estrogen receptor beta, but not estrogen receptor alpha, is present in the vascular endothelium of the human and nonhuman primate endometrium. *J Clin Endocrinol Metab* 2001;**86**:1370–1378.
- Critchley HO, Henderson TA, Kelly RW, Scobie GS, Evans LR, Groome NP, Saunders PT. Wild-type estrogen receptor (ERbeta1) and the splice variant (ERbetacx/beta2) are both expressed within the human endometrium throughout the normal menstrual cycle. *J Clin Endocrinol Metab* 2002;**87**:5265–5273.
- Dubik D and Shiu RP. Mechanism of estrogen activation of c-myc oncogene expression. *Oncogene* 1992;**7**:1587–1594.
- Duyao MP, Buckler AJ, Sonenshein GE. Interaction of an NF-kappa B-like factor with a site upstream of the c-myc promoter. *Proc Natl Acad Sci USA* 1990a;**87**:4727–4731.
- Duyao MP, Kessler DJ, Spicer DB, Sonenshein GE. Binding of NF-KB-like factors to regulatory sequences of the c-myc gene. *Curr Top Microbiol Immunol* 1990b;**166**:211–220.
- Evans MJ, Eckert A, Lai K, Adelman SJ, Harnish DC. Reciprocal antagonism between estrogen receptor and NF-kappaB activity *in vivo*. *Circ Res* 2001;**89**:823–830.
- Fazleabas AT, Kim JJ, Strakova Z. Implantation: embryonic signals and the modulation of the uterine environment—a review. *Placenta* 2004;**25**:S26–S31.
- Feldman I, Feldman GM, Mobarak C, Dunkelberg JC, Leslie KK. Identification of proteins within the nuclear factor-kappa B transcriptional complex including estrogen receptor-alpha. *Am J Obstet Gynecol* 2007;**196**:394e1–311; discussion 394 e11–3.
- Frasor J, Weaver AE, Pradhan M, Mehta K. Synergistic up-regulation of prostaglandin E synthase expression in breast cancer cells by 17beta-estradiol and proinflammatory cytokines. *Endocrinology* 2008;**149**:6272–6279.
- Gruber CJ, Gruber DM, Gruber IM, Wieser F, Huber JC. Anatomy of the estrogen response element. *Trends Endocrinol Metab* 2004;**15**:73–78.
- Guttridge DC, Albanese C, Reuther JY, Pestell RG, Baldwin AS Jr. NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol Cell Biol* 1999;**19**:5785–5799.
- Guzeloglu-Kayisli O, Halis G, Taskiran S, Kayisli UA, Arici A. DNA-binding ability of NF-kappaB is affected differently by ERalpha and ERbeta and its activation results in inhibition of estrogen responsiveness. *Reprod Sci* 2008;**15**:493–505.
- Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* 1999;**140**:5566–5578.
- Harnish DC. Estrogen receptor ligands in the control of pathogenic inflammation. *Curr Opin Investig Drugs* 2006;**7**:997–1001.



- Harnish DC, Scicchitano MS, Adelman SJ, Lyttle CR, Karathanasis SK. The role of CBP in estrogen receptor cross-talk with nuclear factor-kappaB in HepG2 cells. *Endocrinology* 2000;**141**:3403–3411.
- Henderson TA, Saunders PT, Moffett-King A, Groome NP, Critchley HO. Steroid receptor expression in uterine natural killer cells. *J Clin Endocrinol Metab* 2003;**88**:440–449.
- Henriksen PA, Hitt M, Xing Z, Wang J, Haslett C, Riemersma RA, Webb DJ, Kotelevtsev YV, Sallenave JM. Adenoviral gene delivery of elafin and secretory leukocyte protease inhibitor attenuates NF-kappa B-dependent inflammatory responses of human endothelial cells and macrophages to atherogenic stimuli. *J Immunol* 2004;**172**:4535–4544.
- Hombach-Klonisch S, Kehlen A, Fowler PA, Huppertz B, Jugert JF, Bischoff G, Schluter E, Buchmann J, Klonisch T. Regulation of functional steroid receptors and ligand-induced responses in telomerase-immortalized human endometrial epithelial cells. *J Mol Endocrinol* 2005;**34**:517–534.
- Jabbour HN, Kelly RW, Fraser HM, Critchley HO. Endocrine regulation of menstruation. *Endocr Rev* 2006;**27**:17–46.
- Jobin C, Haskill S, Mayer L, Panja A, Sartor RB. Evidence for altered regulation of I kappa B alpha degradation in human colonic epithelial cells. *J Immunol* 1997;**158**:226–234.
- Jones RL, Kelly RW, Critchley HO. Chemokine and cyclooxygenase-2 expression in human endometrium coincides with leukocyte accumulation. *Hum Reprod* 1997;**12**:1300–1306.
- Kennedy TG, Gillio-Meina C, Phang SH. Prostaglandins and the initiation of blastocyst implantation and decidualization. *Reproduction* 2007;**134**:635–643.
- King AE, Critchley HO, Kelly RW. The NF-kappaB pathway in human endometrium and first trimester decidua. *Mol Hum Reprod* 2001;**7**:175–183.
- Kraichely DM, Sun J, Katzenellenbogen JA, Katzenellenbogen BS. Conformational changes and coactivator recruitment by novel ligands for estrogen receptor-alpha and estrogen receptor-beta: correlations with biological character and distinct differences among SRC coactivator family members. *Endocrinology* 2000;**141**:3534–3545.
- Laird SM, Tuckerman EM, Cork BA, Li TC. Expression of nuclear factor kappa B in human endometrium; role in the control of interleukin 6 and leukaemia inhibitory factor production. *Mol Hum Reprod* 2000;**6**:34–40.
- Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL, McCarty KS Jr. Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. *J Clin Endocrinol Metab* 1988;**67**:334–340.
- Marions L, Danielsson KG. Expression of cyclo-oxygenase in human endometrium during the implantation period. *Mol Hum Reprod* 1999;**5**:961–965.
- Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS, Katzenellenbogen JA. Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *J Med Chem* 2001;**44**:4230–4251.
- Milne SA, Perchick GB, Boddy SC, Jabbour HN. Expression, localization, and signaling of PGE(2) and EP2/EP4 receptors in human nonpregnant endometrium across the menstrual cycle. *J Clin Endocrinol Metab* 2001;**86**:4453–4459.
- Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ Jr, Sledge GW Jr. Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. *Mol Cell Biol* 1997;**17**:3629–3639.
- Nishida M, Kasahara K, Kaneko M, Iwasaki H, Hayashi K. Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors. *Nippon Sanka Fujinka Gakkai Zasshi* 1985;**37**:1103–1111.
- Noyes RW. Dating the endometrial biopsy. *Fertil Steril* 1950;**1**:3–25.
- Page M, Tuckerman EM, Li TC, Laird SM. Expression of nuclear factor kappa B components in human endometrium. *J Reprod Immunol* 2002;**54**:1–13.
- Petz LN, Ziegler YS, Schultz JR, Kim H, Kemper JK, Nardulli AM. Differential regulation of the human progesterone receptor gene through an estrogen response element half site and Sp1 sites. *J Steroid Biochem Mol Biol* 2004;**88**:113–122.
- Quaedackers ME, van den Brink CE, van der Saag PT, Tertoolen LG. Direct interaction between estrogen receptor alpha and NF-kappaB in the nucleus of living cells. *Mol Cell Endocrinol* 2007;**273**:42–50.
- Rubio MF, Werbajh S, Cafferata EG, Quagliano A, Colo GP, Nojek IM, Kordon EC, Nahmod VE, Costas MA. TNF-alpha enhances estrogen-induced cell proliferation of estrogen-dependent breast tumor cells through a complex containing nuclear factor-kappa B. *Oncogene* 2006;**25**:1367–1377.
- Saitoh M, Ohmichi M, Takahashi K, Kawagoe J, Ohta T, Doshida M, Takahashi T, Igarashi H, Mori-Abe A, Du B et al. Medroxyprogesterone acetate induces cell proliferation through up-regulation of cyclin D1 expression via phosphatidylinositol 3-kinase/Akt/nuclear factor-kappaB cascade in human breast cancer cells. *Endocrinology* 2005;**146**:4917–4925.
- Simon C, Frances A, Piquette GN, el Danasouri I, Zurawski G, Dang W, Polan ML. Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist. *Endocrinology* 1994;**134**:521–528.
- Simon C, Mercader A, Gimeno MJ, Pellicer A. The interleukin-1 system and human implantation. *Am J Reprod Immunol* 1997;**37**:64–72.
- Speir E, Yu ZX, Takeda K, Ferrans VJ, Cannon RO 3rd. Competition for p300 regulates transcription by estrogen receptors and nuclear factor-kappaB in human coronary smooth muscle cells. *Circ Res* 2000;**87**:1006–1011.
- Sun WH, Keller ET, Stebler BS, Ershler WB. Estrogen inhibits phorbol ester-induced I kappa B alpha transcription and protein degradation. *Biochem Biophys Res Commun* 1998;**244**:691–695.
- Takebayashi T, Higashi H, Sudo H, Ozawa H, Suzuki E, Shirado O, Katoh H, Hatakeyama M. NF-kappa B-dependent induction of cyclin D1 by retinoblastoma protein (pRB) family proteins and tumor-derived pRB mutants. *J Biol Chem* 2003;**278**:14897–14905.
- Tranguch S, Daikoku T, Guo Y, Wang H, Dey SK. Molecular complexity in establishing uterine receptivity and implantation. *Cell Mol Life Sci* 2005;**62**:1964–1973.
- van Mourik MS, Macklon NS, Heijnen CJ. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. *J Leukoc Biol* 2009;**85**:4–19.
- Wang H, Critchley HO, Kelly RW, Shen D, Baird DT. Progesterone receptor subtype B is differentially regulated in human endometrial stroma. *Mol Hum Reprod* 1998;**4**:407–412.
- Wissink S, van der Burg B, Katzenellenbogen BS, van der Saag PT. Synergistic activation of the serotonin-1A receptor by nuclear factor-kappa B and estrogen. *Mol Endocrinol* 2001;**15**:543–552.

Submitted on September 22, 2009; resubmitted on November 1, 2009; accepted on November 4, 2009