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Expression of the epidermal growth factor system in human endometrium during the menstrual cycle

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The epidermal growth factor (EGF) system is ubiquitous in humans and plays fundamental roles in embryogenesis, development, proliferation and differentiation. As the endometrium of fertile women is characterized by proliferation and differentiation, we hypothesize a role for the EGF system. Fourteen premenopausal women had endometrial samples removed on day 6 ± 1 and day 6 ± 1 and 12 ± 1 after ovulation during one menstrual cycle. RNA was extracted and analysed by real-time PCR, and immunohistochemistry was performed to localize the components of the EGF system. Human EGF Receptor 1 (HER1) showed highest expression during the proliferative phase, HER2 and HER4 during the early and HER3 during the late secretory phase. Amphiregulin (AR) and transforming growth factor alpha (TGFα) expression is highest in proliferative phase. Heparin binding (HB)-EGF and betacellulin (BCL) show no variation. Epiregulin (EP) is detectable in some samples. EGF is undetectable. HER1, HER2, HER3 and HER4 were localized to the epithelium and glands HER3 and HER4 solely in the secretory phase. Amphiregulin was seen in leucocytes and stromal cells, TGFα and betacellulin in the epithelial lining, epiregulin in stromal cells whereas HB-EGF and EGF are undetectable. In conclusions, we observed cyclical expression of the four EGF receptors and two ligands and localized all four receptors and four ligands in endometrial biopsies. This suggests a role for the EGF system in growth of the endometrium.

Key words: EGF receptors/epidermal growth factor/human endometrium/menstrual cycle

Introduction

The epidermal growth factor (EGF) system is a type I growth factor family consisting of four receptors: Human EGF Receptor 1 (HER1) (also called EGF-receptor/EGFr/ErbB1), HER2 (also called ErbB2), HER3 and HER4. The receptors are transmembrane glycoproteins with an extracellular ligand-binding domain, a transmembrane region and an intracellular domain. The intracellular domains of HER1, HER2 and HER4 display tyrosine kinase activity. Activation of the receptors induces dimerization. HER1 and HER4 form either homo- or heterodimers, whereas HER2 functions as a cofactor for the other receptors, and HER3 needs heterodimerization because of its lack of tyrosine kinase activity (Figure 1). Many ligands, or EGF-related peptide growth factors, bind to the receptors. One group consists of EGF, amphiregulin (AR), transforming growth factor alpha (TGFα), heparin binding EGF like growth factor (HB-EGF), betacellulin (BCL), and epiregulin (EPI). These bind to HER1. HB-EGF, EPI and BCL also bind to HER4 (Figure 1). The EGF system is ubiquitous in human organs and plays fundamental roles in diverse processes such as embryogenesis, development, proliferation and differentiation (Carpenter, 2003; Citri *et al*., 2003; Harris *et al*., 2003). The endometrium is characterized by ongoing proliferation and differentiation throughout almost half of a woman's life, and it is tempting to hypothesize that the EGF system plays a pivotal role in controlling these reactions.

The endometrium is unique as during the fertile period of a woman's life it undergoes cyclic variation and is shed at menstruation. In the first half of the menstrual cycle the functional layer of the endometrium, consisting of epithelial and stromal cells, is proliferating. There is an overlap between menstruation and the proliferative phase; the proliferative phase begins before menstruation has ceased and is followed by the secretory phase. Now the epithelial glands of the endometrium become more secretory and prepare for nidation of the blastocyst.

Only a limited number of studies concerning the EGF system and the endometrium have been published. In these studies one or a few members of the EGF system have been investigated (Berchuck *et al*., 1991; Prigent *et al*., 1992; Imai *et al*., 1995; Miturski *et al*., 1998; Srinivasan *et al*., 1999; Chobotova *et al*., 2002). Most of these studies are based on immunohistochemistry of single biopsies from women undergoing hysterectomy on benign indications.

This study describes the expression and the localization of the receptors and ligands of the EGF system in endometrial samples taken from healthy, fertile women on three occasions during one menstrual cycle. The samples were investigated by real-time PCR and immunohistochemistry.

Material and methods

The inclusion criteria were age between 18 and 41 years; Caucasian; mother tongue Danish; at least two ordinary menstrual bleedings after latest pregnancy, cessation of breast feeding, medical treatment, removal of intra uterine device or cessation of hormonal contraception, body mass index (BMI)

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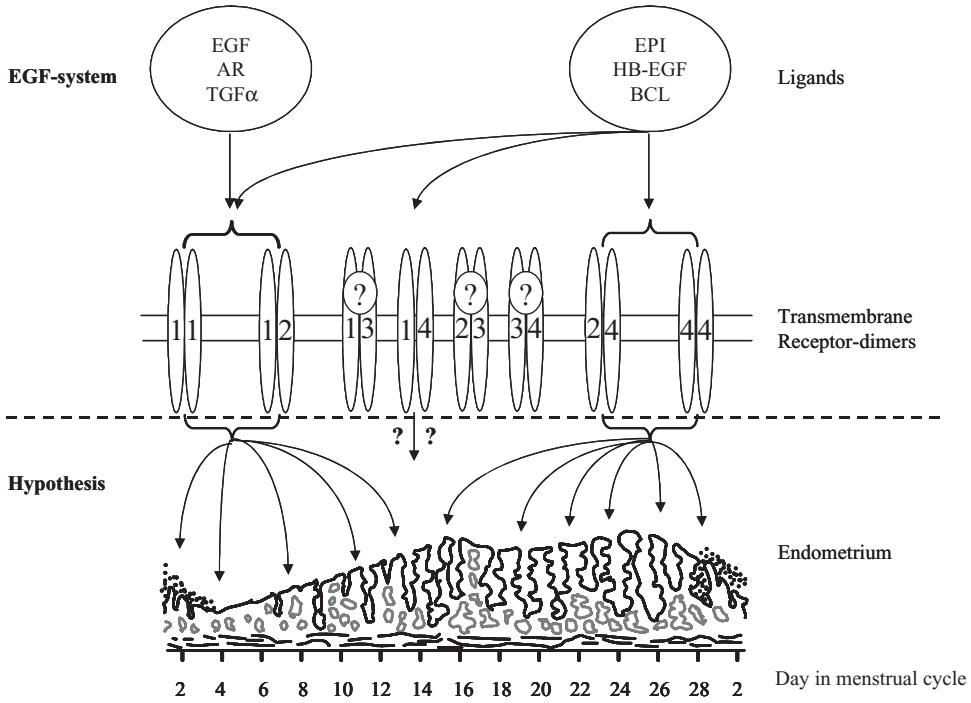

Figure 1. The investigated receptors and ligands of epidermal growth factor (EGF) system and hypothesis of the action of the EGF system during the menstrual cycle. Above the dashed line: The EGF system and the binding specificities of the EGF ligands. HER1, HER2 and HER4 form hetero- or homodimers, HER3 forms a heterodimer. EGF, AR and TGFα bind to HER1. HB-EGF and EPI and BCL bind to HER1 and HER4. No ligand is identified for HER2, HER2 functions as a cofactor for the other receptors. ?: Ligands for HER3 were not investigated. Below the dashed line: The hypothesis of the role of the EGF system. The proliferative agents, HER1 and EGF, AR and TGFα, exert their main influence during the menstrual and proliferative phases. The differentiating, anti-proliferative and apoptotic agents, HER3, HER4 and their ligands, are the main actors during the secretory phase. HER2 serves as the preferred co-factor for the other receptors. ? indicates that time for the main influence of the heterodimer HER1/HER4 is debatable.

between 20 and 30, no history of former pathology concerning the endometrium or myometrium and a minimum of one vaginal childbirth.

Fourteen women were included. The data for these women were median age in years, 34.5 (range 24.2–40.7) and BMI, 24 (range 20–29). The length of the menstrual cycle was on average 29.4 days (range 24–38) in more than two menstrual cycles before the menstrual cycle in which the samples were taken. The length of investigational menstrual cycle was 28.6 days (range 22–42).

The women were included from January 2002 to April 2003. All examinations on each woman took place during one menstrual cycle. The women were examined by transvaginal ultra sound examination (ULS) and had an endometrial sample and a peripheral blood sample taken. Samples were planned on day 6 ± 1 (early proliferative phase) after the first day of menstruation (day 6 post menstruation, day 6 p.m.) and at day 6 ± 1 (early secretory phase) (day 6 post ovulation, day 6 p.o.) and at day 12 ± 1 (late proliferative phase) (day 12 post ovulation, day 12 p.o.) after ovulation. The actual investigations took place at day 6.6, (range 5–8) (early proliferative phase), day 6.4 p.o. (range 5–8) (early secretory phase) and day 12.7 p.o. (range 12–14) (late secretory phase). The day of ovulation was detected by use of ClearPlan®, (Unipath Limited, Bedford, England). This ovulation-test is based on the peak in luteinizing hormone in the urine 24–36 h before ovulation. The ultrasound examinations were performed to ensure normal internal genitals and were performed using either an Acuson Sequoia 512 or a Siemens ultrasound Scan Machine (Siemens, Ballerup, Denmark). The endometrial samples were taken using a uterine explora curette® (Milex Products Inc., Chicago, IL, USA); half of the tissue was snap frozen in liquid nitrogen and stored at −80°C, the other half was kept in Lillie's liquid®, a formaldehyde buffer 4% neutral and with pH 7.0 (Merck Eurolab A/S, Glostrup, Denmark).

All investigations followed the Declaration of Helsinki, and all participants gave informed consent before participation. The study was approved by the local scientific ethic committee (journal number 2001–0253).

RNA extraction

Total RNA was extracted from endometrial samples using QIAamp RNA Blood kit® (Qiagen, Merck Eurolab A/S), and all samples were DNase treated using the RNase-Free DNase Set® (Qiagen, Merck Eurolab A/S). Instructions given by the company were followed. After purification the concentration and purity of the RNA preparations were analysed by optical density at wavelengths of 260 and 280 nm by use of a GeneQuant II® (Pharmacia Biotech, Cambridge, England).

Reverse transcription

The RNA solutions were diluted to 0.1 µg/ml. 0.1 µg was mixed with 5 µl $MgCl_2$ 25 mM, (Roche A/S, Hvidovre, Denmark), 2 µl 10 × PCR buffer II (Roche A/S), 1 µl Oligo $d(T)_{16}$ 50 µM (DNA Technology A/S, Århus, Denmark), 8 µl dNTPmix (x µl dATP/dTTP/dCTP/dGTP) (Pharmacia Biotech, Hilleroed, Denmark), 1 µl MuLV (Moloney murine Leukaemia Virus) reverse transcriptase, (50 U/ml) (PerkinElmer Denmark A/S, Hvidovre, Denmark), 1 µl RNase inhibitor, (20 U/ml) (PerkinElmer Denmark A/S) and 1 µl water. cDNA was synthesized by incubation at 42°C for 30 min and the process was stopped by 99°C for 5 min. cDNA was either used immediately or stored at −20°C.

Real-time PCR

Real-time PCR was performed by use of a LightCycler (Roche A/S). The PCR mix was made on the basis of the prescription from the supplier: 5.4 µl sterile water, 1.6 µl $MgCl_2$ (25 mM) (Roche A/S), 0.5 µl sense and 0.5 µl antisense primers (see Table I for concentrations and primer sequences), 1.0 µl SYBRgreen (Roche A/S) and 1.0 µl target cDNA in a total volume of 20 µl. RNA extracted from a bladder cancer cell line, HCV29 (ATCC, Manassas, Virginia, USA), was used as a calibrator control for HER1, HER2, EGF, AR, TGFα, HB-EGF and EPI, whereas an endometrial cancer cell line, HEC 1A (ATCC), was used as calibrator for HER3, and another endometrial cancer cell line, KLE (ATCC), was used as calibrator for HER4. HER2 was analysed in a real time reaction employing probes according to the Fluorescence Resonance Energy Transfer (FRET) principle: The PCR mix was made according to the prescription from Roche: 2.0 µl probe mix (Roche A/S), 1.0 µl of the probe (1 pmol/ml), 1.0 µl sense (5 pmol/ml), 1.0 µl antisense (5 pmol/ml), 3.2 µl 25 mM MgCl, 9.8 µl sterile water and 2.0 µl cDNA. The PCR reactions were run by an

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Table I. Primer concentration, annealing temperatures, primer sequences and sizes of amplified cDNA fragments

Target	Concentration pmol/ml	Annealing temperature	Primer sequence	cDNA size*
HER1	5	57	s: GAGAGGAGAACTGCCAGAA as: GTAGCATTTATGGAGAGTG	454
HER2	5	59	s: CCAGGACCTGCTGAACTGGT as: TGTACGAGCCGCACATCC	272
HER3	5	65	s: GGTGCTGGGCTTGCTTTT as: CGTGGCTGGAGTTGGTGTTA	365
HER4	5	65	s: TGTGAGAAGATGGAAGATGGC as: GTTGTGGTAAAGTGGAATGGC	265
AR	10	58	s: GGCTCAGGCCATTATGC as: ACCTGTTCAACTCTGACTGA	266
TGFα	10	70	s: GCCCGCCCGTAAAATGGTCCCCTC as:GTCCACCTGGCCAAACTCCTCCTCTGGG	528
HB-EGF	10	58	s: GTGCCTAGACTGTTACTTTG as: GAAATGTAGACAGACATTAAAT	126
EGF	10	54	s: AGCAATTGGTGGTGGATG as: ACTCTTTGCAAAAGTTGTC	103
BCL	10	66	s: TCTAGGTGCCCCAAG C as: GTGCAGACACCGATGA	220
EPI	10	60	s: CAAAGTGTAGCTCTGACATG as: CTGTACCATCTGCAGAAATA	238

AR, amphiregulin; BCL, betacellulin; EGF, epidermal growth factor; EPI, epiregulin; HB-EGF, heparin binding-EGF; HER1, Human EGF Receptor 1; TGFα, transforming growth factor alpha.
*Size of amplified cDNA fragment.

initial denaturation step at 95°C for 30 s and then the number of cycles indicated in Table I, with a 95°C denaturation followed by annealing (see temperature in Table I) for 5 s and 72°C extension for 10 s. A standard melting curve was used to check the quality of amplification. A calibration curve and positive and negative controls were included in each run. The calibration curve was composed of serial dilutions of a pool of RNA from cultured cell lines RNA for each of the mRNAs examined. Generating the calibration curve the following concentrations were used: 1; 0.5; 0.25; 0.0625; 0.03125; 0.0156 and 0.0078 μg/ml. The calibrator concentration 0.0125 μg/ml served as positive control, whereas as a negative control water was added instead of RNA. All samples from the same women were analysed in the same run.

The imprecision was calculated based on samples run in the same run (n = 23) and on samples run over a period of 37 months (n = 13–37). Based on results obtained for HER1-4 and the ligands AR, TGFα, HB-EGF, BCL and EPI the intraassay variation was 11–19% and the interassay variation was 15–27%.

The results are presented as absolute values relative to the mRNA content in the calibrator used for generating the calibration curve. The levels of expression of each component are not comparable, because the absolute amount of mRNA for each species examined is unknown.

Initially we wanted to use a housekeeping gene as a reference, but found significant cyclic variation during the menstrual cycle for both $\beta_2$microglobulin and 18SRNA (data not shown). From the literature, it has been shown that β-actin also displays this cyclic variation (Sahlin, 1995).

Immunohistochemical analysis

Tissues were fixed in 4% buffered paraformaldehyde (Merck Eurolab A/S) for 24 h, embedded in paraffin, and cut into 10-μm sections using a microtome. After incubation for 30 min in 10% normal rabbit serum (code number X0902; Dako, Copenhagen, Denmark), sections were incubated for 18 h at room temperature with the primary antisera; AR (ab 1, polyclonal; Neomarkers, Fremont, California, USA), TGFα (AB 1, monoclonal; Oncogene, Merck KGaA, Darmstadt, Germany), BCL (AB-261-NA, polyclonal; RD, Oxon), HER1 (E30, monoclonal; Oncogene, Merck KGaA), HER2 (A0485; Dako), HER3 (AB10, polyclonal; Neomarkers) and HER4 (Ab-4 Clone HFR-1; Neomarkers). The primary antisera were diluted 1/100 and 1/400. The immunoreaction was visualized by means of biotinylated rabbit anti-mouse immunoglobulins (code number 0354, monoclonal antibodies; Dako) or biotinylated swine anti-rabbit immunoglobulins (code number E353, polyclonal antibodies; Dako) diluted 1/40 for 1 h as the second layer, followed by StreptABComplex/horseradish peroxidase (code number K0377; Dako) diluted 1/100 as the third layer, and finally staining by means of 3,3-diaminobenzidine for 30 min. Sections were counterstained with hematoxylin. For controls, the sections were incubated with nonimmune serum, and similar sections with immunoreactions to the various ligands were compared to exclude nonspecific reactions.

The blood samples were analysed (serum) employing ADVIA Centaur (Bayer, Kgs. Lyngby, Denmark) and the assays for FSH, LH, progesterone and estradiol supplied by the manufacturer (Bayer).

Statistics

Wilcoxon test, a nonparametric test for two related samples, was used to show the relation between samples from each woman. SPSS 10.0 for Windows and GraphPad 3.03 were used for statistical investigations and making relevant figures.

Results

The variation of the receptor and ligand expression in endometrial biopsies obtained on three occasions during the menstrual cycle is shown in Figures 2 and 3.

HER1 (Figures 2A and 3A) shows its highest mRNA expression in the early proliferative phase, HER2 (Figures 2B and 3B) and HER4 (Figures 2D and 3D) in the early secretory phase and HER3 (Figures 2C and 3C) during the secretory phase. The expression of HER1 (Figures 2A) is lowest during the secretory phase, whereas HER2 (Figure 2B) expression is low from the late secretory to the early proliferative phase. HER3 (Figure 2C) expression is low in the early proliferative phase, whereas HER4 (Figure 2D) expression is low in the late secretory phase.

From the early proliferative to the early secretory phase both the expression of AR (Figures 2E and 3E) and TGFα (Figures 2F and 3F) decrease significantly. TGFα (Figure 2F) increases during the secretory phase to reach a maximum in the late secretory phase. HB-EGF (Figures 2G and 3G) and BCL (Figures 2H and 3H) show no variation during the menstrual cycle. EPI is detectable in 5 of 14 samples in the early proliferative, 3 of 14 samples in the early secretory and 10 of 14 samples in the late secretory phase. EGF is not detectable in any of the samples.

By immunohistochemistry it was possible to identify the receptors, HER1, HER2, HER3 and HER4. HER1 is solely localized basally in the luminal surface epithelium and in the glands situated close to the

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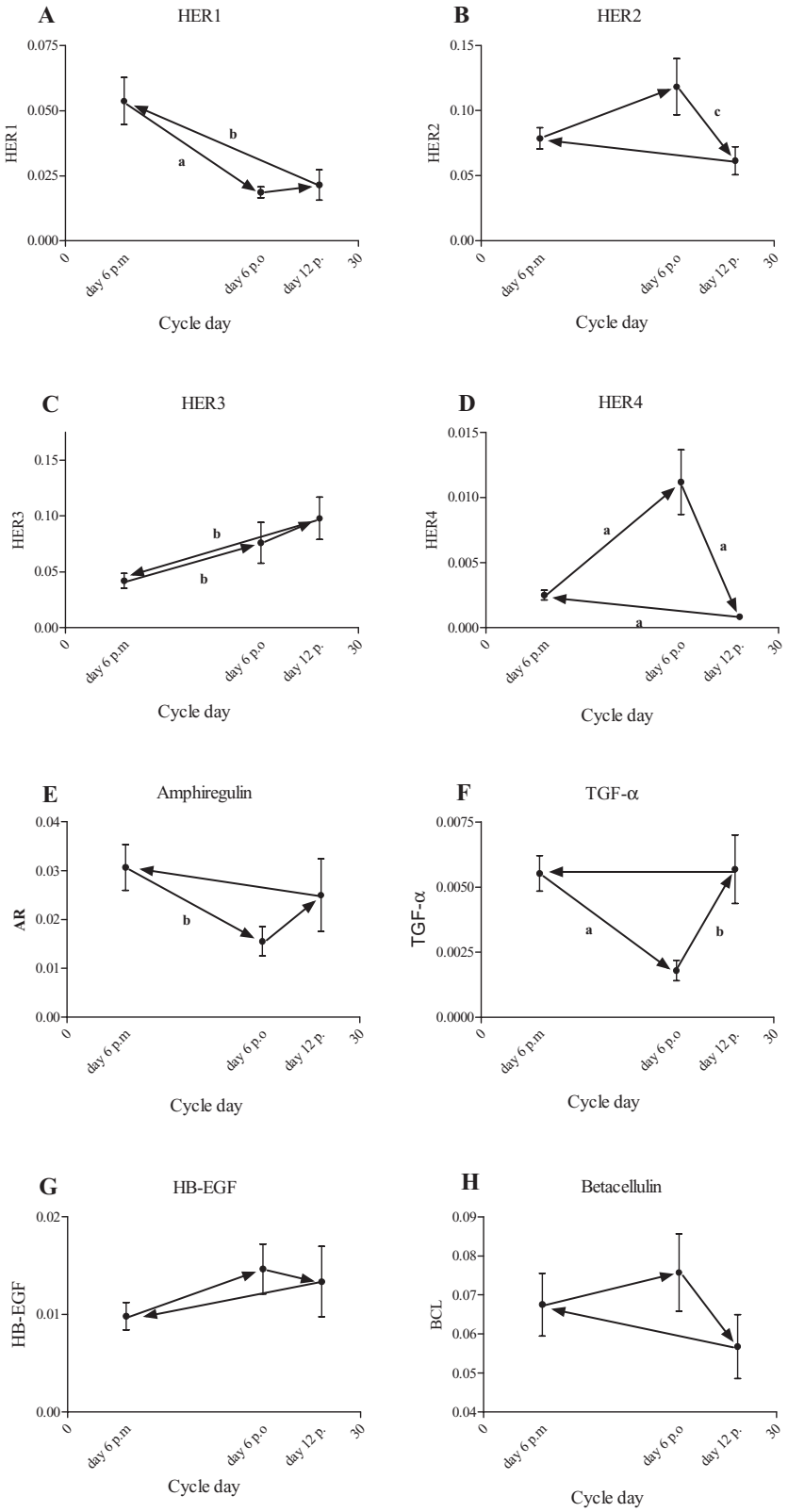

Figure 2. mRNA expression of the receptors, (**A**) HER1, (**B**) HER2, (**C**) HER3 and (**D**) HER4 and four ligands, (**E**) amphiregulin, AR, (**F**) transforming growth factor alpha, TGFα, (**G**) heparin binding (HB)-epidermal growth factor (EGF) and (**H**) betacellulin, BCL in endometrial biopsies from 14 women removed on day 6 post menstruation (p.m.), day 6 post ovulation (p.o.) and day 12 p.o. Day 6 p.m. represent day 6 after first day of menstruation, day 6 p.o and day 12 p.o. day 6 and day 12 after ovulation, respectively. Y-axes are arbitrary units and individual for each component. The expressions are given as mean and standard error of mean. Arrows indicate time direction. Significant differences are indicated as **a**, $P < 0.001$; **b**, $P < 0.01$ and **c**, $P < 0.05$.

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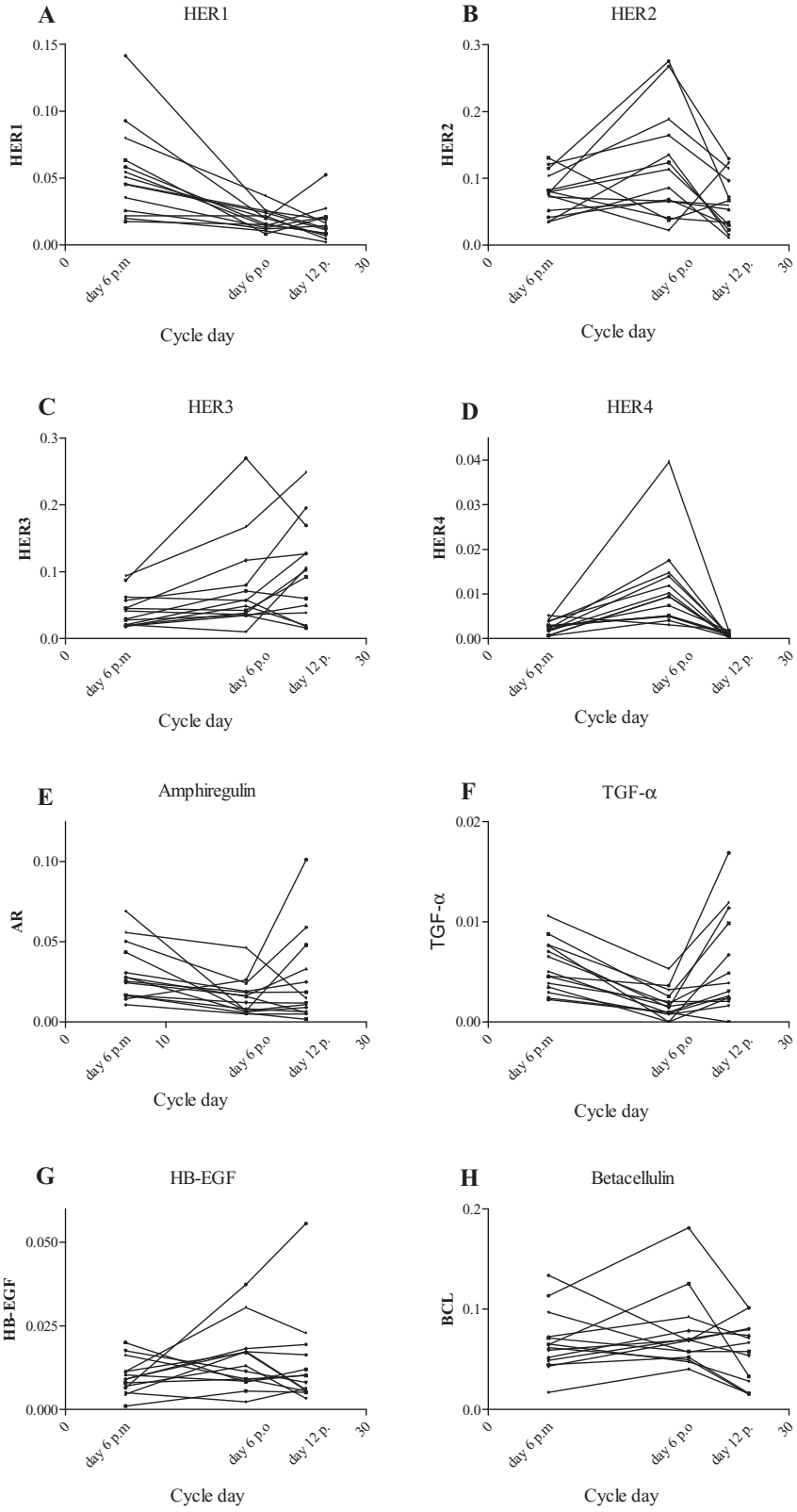

Figure 3. mRNA expression of the receptors, (**A**) HER1, (**B**) HER2, (**C**) HER3 and (**D**) HER4 and four ligands, (**E**) amphiregulin, AR, (**F**) transforming growth factor alpha, TGFα, (**G**) heparin binding (HB)-epidermal growth factor (EGF) (**H**) and betacellulin, BCL in endometrial biopsies from 14 women removed on day 6 p.m., day 6 p.o and day 12 p.o. Day 6 p.m. represent day 6 after first day of menstruation, day 6 p.o and day 12 p.o. day 6 and day 12 after ovulation, respectively. Y-axes are arbitrary units, and individual for each component. Expression throughout the menstrual cycle for each woman is given by symbols connected by a line.

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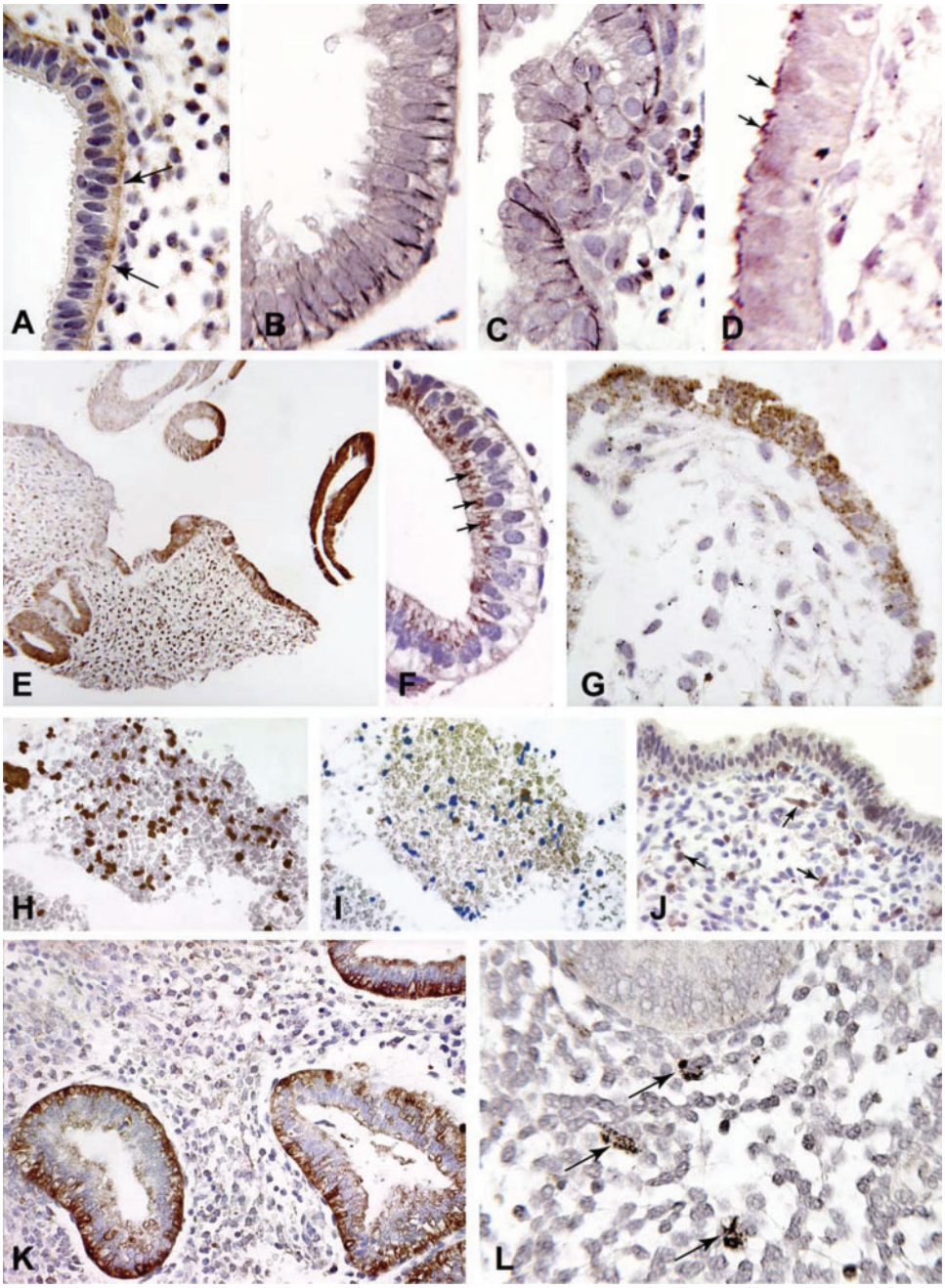

Figure 4. Localizations of the epidermal growth factor (EGF) receptors and ligands identified by immunohistochemical methods. (**A**) HER1 immunoreactivity localized to the basal part of the luminal surface epithelium (arrows), late secretory phase. (**B**) and (**C**) HER2 immunoreactivity localized to the baso-lateral membrane of the epithelium and glands, **B** is early secretory phase, **C** is late secretory phase. (**D**) HER3 immunoreactivity seen as a rim localized to the apical surface of the luminal epithelial cells (arrows), early secretory phase. (**E**) HER4 seen as a staining of the cytoplasm of epithelial and glandular cells, early secretory phase. (**F**) transforming growth factor alpha (TGFα) in early secretory phase localized luminal to the nuclei of the glandular cells (arrows) and as cytoplasmic granules in the late secretory phase, (**G**). Immunoreactivity to AR was present mainly in leucocytes of the blood, (**H**), late secretory phase. In **I** amphiregulin (AR) antiserum was replaced by nonimmune serum for comparison as a control, also late secretory phase. Nuclei of leucocytes are stained blue by hematoxylin. (**J**) Amphiregulin (AR) immunoreactivity in a few stromal cells (arrows), late secretory phase. (**K**) Betacellulin (BCL) staining in the basal part of the glandular cells in the early secretory phase and **L** epiregulin (EPI) in a few stromal cells (arrows), proliferative phase. Magnification: **A** and **F** ×400, **B** and **C** ×550, **D** and **L** ×600, **E** ×100, **G** ×500, **H**–**K** ×300.

surface (Figure 4A). The reaction was strongest in the surface epithelium and there was no staining of the basal parts of the glands or the stroma. There was no obvious difference in intensity dependent on the menstrual cycle. HER2 reaction is clearly visible baso-lateral in the glands and surface epithelium, and the staining in the glands is stronger compared to the surface epithelium (Figure 4B and C). Staining with the HER3 antibody showed a faint staining localized to the luminal rim of the surface epithelial cells in the secretory phase (Figure 4D). HER4 was seen in the cytoplasma of some parts of the glandular and surface epithelium in the early or late secretory phase (Figure 4E).

The ligand TGFα was seen in all three phases in the surface epithelium, in the early secretory phase luminal to the nucleus (Figure 4F) and in the late secretory phase in cytoplasmic granules (Figure 4G).

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AR was not detectable in the epithelium or the glands, but stains in leucocytes in blood, which was present in varying amounts in the specimens, especially in the secretory phase (Figure 4H and I). AR was also detected in a few stromal cells in the tissue (Figure 4J). BCL was localized to the basal part of the glandular cells and not in the stroma (Figure 4K), whereas epiregulin was present in scattered stromal cells and not in the epithelium (Figure 4L). With the present antibodies and techniques we did not detect EGF and HB-EGF in the endometrium.

Serum analyses of LH, FSH, progesterone and estradiol verified normal menstrual cycles and premenstrual status of the women participating in the study (data not shown).

Discussion

We report variation in the expression of mRNA in all four receptors and two of the ligands of the EGF system in the endometrium during the menstrual cycle and show that the receptors and majority of the ligands detected by mRNA analysis are present also as proteins detected by immunohistochemistry.

The four receptors peak differently during the menstrual cycle. HER1 shows the highest value in early proliferative phase, HER2 and HER4 in early secretory phase, and HER3 in late secretory phase. Two of the ligands, AR and TGFα, show cyclic variation, and both display low levels during the early secretory phase.

We found individual cases where measurements of mRNA expression was opposite of the majority (Figure 3). However taken together the data show a statistical significant variation as a function of the menstrual cycle (Figure 2).

In designing this study, it was necessary to choose the timing of sampling in the menstrual cycle. One sample in both proliferative and secretory phase seemed obvious. The proper time for the third sample is more debatable. We chose to get two samples during the secretory phase, to get information about the diffentiation the endometrium is undergoing, the first at day 6 after ovulation to be as near as possible to the optimal time for nidation of the blastocyst, and yet without inflicting damage to the endometrium (Johannisson *et al*., 1981) before the last sampling in late secretory phase at day 12 after ovulation. The first sample was chosen as an early proliferative phase sample which we expected to ensure proliferative status, and the last to be as close to menstruation, and yet not get a partly or fully degraded sample. Our above-mentioned considerations and decisions do not give us certainty that we reach the peak of expression for the factors investigated. A peak in expression of some components might be hidden by the sampling procedure, in the same way as would be the case for sex-hormones (Juul *et al*., 1997).

In former studies the receptors of the EGF system, HER1, HER2, HER3 and HER4, have been examined mostly by immunohistochemistry and are typically based on few, non consecutive samples. These studies show conflicting data concerning variation during the menstrual cycle and localization (Taketani and Mizuno, 1988; Troche *et al*., 1991; Bigsby *et al*., 1992; Prigent *et al*., 1992; Wang *et al*., 1994; Imai *et al*., 1995; Konopka *et al*., 1998; Dahmoun *et al*., 1999; Srinivasan *et al*., 1999). By immunohistochemistry we localized HER1 to the basal part of the surface epithelial cells in all three phases investigated. This is in agreement with some (Bigsby *et al*., 1992; Imai *et al*., 1995), but in contrast to others (Wang *et al*., 1994) who described the EGF-receptor localized only in stromal cells, or both to epithelial and stromal cells (Moller *et al*., 2001). HER2 localized baso-laterally, and solely to the glands and epithelium is in accordance with others findings (Wang *et al*., 1994; Miturski et al., 1998; Bigsby *et al*., 1992), as is HER3 identified in the epithelium (Prigent *et al*., 1992) and in faint staining(Srinivasan *et al*., 1999). HER4 being visible in early and late secretory phase is partly the same as previously demonstrated (Chobotova *et al*., 2005). In the paper by Chobotova *et al*. HER4 was localized mainly to the stroma in the proliferative phase and to the epithelium and stroma in the secretory phase, whereas in this study we localized HER4 to the epithelium. The Chobotova *et al*. paper was based on nine samples from proliferative phase and five samples from secretroy phase, all samples were from women undergoing sterilization or hysterectomy on benign indication The authors report HER4 to be localized mainly to the stroma in the proliferative phase and to the epithelium and stroma in the secretory phase, whereas we localize HER4 to the epithelium only. Another immunohistochemical-based study defines HER4 to both stroma and epithelium and find significantly higher expression in secretory as compared to proliferative phase (Srinivasan *et al*., 1999). A study based on nine endometrial samples divided into three phases investigated by immunohistochemistry and RT–PCR showed the expression of TGFα and EGF to increase from early proliferative to late proliferative phase and decrease from late proliferative phase to secretory phase (Imai *et al*., 1995). A preovulatory peak is in fact not in opposition to our data, because we have no samples around the time of ovulation due to the study design. The TGFα localization granules of the epithelium is in agreement with previous findings (Imai *et al*., 1995; Hansard *et al*., 1997). Others have recently described TGFα mainly to be localized to the epithelium, but also to the stroma (Reis *et al*., 2005). The description of EGF with an epithelial localization (Imai *et al*., 1995) and epithelial and stromal localization (Moller *et al*., 2001; Reis *et al*., 2005) were not confirmable by us, as we were not able to demonstrate the presence of EGF either by immunohistochemistry or RT–PCR. We found AR in a few stromal cells but also in leucocytes. AR has previously been demonstrated in monocytes (Mograbi *et al*., 1997). Some of the AR measured by RT–PCR might originate from blood, and since the content of blood in the samples varies, this also would imply variation in the measured contents. HB-EGF expression investigated by RT–PCR demonstrated the presence of HB-EGF through out the menstrual cycle with no difference between the phases (Birdsall *et al*., 1996), similar to our finding, but in opposition to other studies using other methods (Ebert *et al*., 2000; Chobotova *et al*., 2002; Lessey *et al*., 2002). A study only concerned with the HB-EGF expression at the time of implantation demonstrates a day to day variation reaching a maximum at day 8 after ovulation (Stavreus-Evers *et al*., 2002).

Interpreting our results, we find it important to keep in mind, that our study design is different from others as we have chosen consecutive sampling. This might very well be the reason, why we find cyclical variation by realtime-PCR. Regarding the immunohistochemistry, this part of the study is solely meant for describing the localization. Some of the inconsistency with other studies might be due to different procedures and employing different antibodies.

We demonstrate concordance between mRNA and detectable protein for HER1, HER2, HER3 and HER4 and for AR, TGFα, BCL and EPI. HB-EGF and EGF were not detectable by immunohistochemistry.

Based on our current knowledge one can only speculate on the exact role and function of the EGF system in the endometrium (Figure 1). HER1 and its ligands, EGF, AR and TGFα are proliferative agents (Luetteke *et al*., 1999; Olayioye *et al*., 2000). We therefore hypothesize that HER1 and TGFα control the events taking place from the end of the late secretory to the early secretory phase, in other words the menstrual and proliferative phases (Figure 1). These phases overlap as proliferation starts at the time of shedding of the old endometrium (Bigsby *et al*., 1992). This means that the proliferation beginning somewhere between late secretory and early proliferative phase is concomitant with the increase in HER1 expression and takes place after the increase in TGFα during the secretory phase. HER1,

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AR and TGFα all decrease from early proliferative phase to reach a minimum at early secretory phase. Besides the proliferative stimulus, HER1 stimulates migration and could be involved in migration of the epithelial cells towards the surface in the proliferative phase.

HER2, HER3 and HER4 are likely to exert their main influence in the secretory phase (Figure 2). HER2 is primarily a proliferative agent through dimerization with mainly HER1, but also the preferred co-receptor through dimerization with HER3 and HER4 (Falls, 2003). In general HER3 and HER4 play a role in differentiation, inhibition of proliferation, apoptosis, migration and adhesion (Falls, 2003). The expression of HER2 and HER4 decreases towards the end of the menstrual cycle or at late secretory phase, whereas HER3 decreases during menstruation. HER3 has little or no tyrosine kinase activity (Srinivasan *et al*., 1999) and thus demands heterodimerization with one of the other receptors. We speculate that the decrease in HER2 and HER4 could down-regulate the action of HER3 by preventing dimerization, and thereby be interpreted as a regulating mechanism of HER3. During the menstrual phase, HER4 may act as a regulating proliferative inhibitor of a strong proliferative stimulus exerted through HER1.

Beside the proliferative action, the increase in TGFα expression in the late secretory phase might also represent a cell survival mechanism, an attempt to avoid apoptosis, as described in a study suggesting TGFα as an anti-apoptotic ligand (Yarden, 2001). Apoptosis and proliferation were studied in the endometrium from day 4 before menstruation to day 2 of the menstrual phase in epithelium and stroma by immunohistochemistry. The apoptotic index increased from day 4 in the epithelium and peaked at day 2 in the menstrual phase (Dahmoun *et al*., 1999).

HB-EGF works through HER1 or HER4 and exerts its effect at the site of nidation of the fertilized ovum probably as a potential paracrine factor during implantation (Das *et al*., 1994; Olayioye *et al*., 2000; Lessey *et al*., 2002). In this context HER4 has been interpreted as a high-affinity receptor on the surface of implantation-competent blastocysts (Paria *et al*., 1999). The expression of HB-EGF showed no change over the three phases, suggesting that HB-EGF needs a stimulus only present if the woman becomes pregnant.

EPI was detectable in some of the samples, mostly from the late secretory phase. This gives a cyclical tendency. The low expression is in accordance with general descriptions of EPI as a growth factor predominantly expressed in tumour cells (Toyoda *et al*., 1997; Shelly *et al*., 1998).

In conclusion we find cyclic variation of the expression of the four EGF system receptors, HER1, HER2, HER3 and HER4, and two ligands, AR and TGFα. This strongly indicates a central role of the EGF system in the regulation of the cyclical growth and shedding of the human endometrium.

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