Endometrial expression of the estrogen-sensitive genes MMP-26 and TIMP-4 is altered by a substitution protocol without down-regulation in IVF patients

R.Pilka¹,², I.Oborna¹, V.Lichnovsky³, P.Havelka³, H.Fingerova¹, P.Eriksson⁴, S.Hansson² and B.Casslén²,⁵

¹Department of Obstetrics and Gynaecology, Palacky University, Olomouc, Czech Republic, ²Department of Obstetrics and Gynaecology, University Hospital, Lund, Sweden, ³Department of Histology and Embryology, Palacky University, Olomouc, Czech Republic and ⁴Atherosclerosis Research Unit, King Gustav V Research Institute, Karolinska Hospital, Stockholm, Sweden

5To whom correspondence should be addressed at: Biomedical centre C 14, Lund, S-221 84 Sweden. E-mail: bertil.casslen@gyn.lu.se

BACKGROUND: The aim of this study was to analyse the effects of an estradiol (E₂)–progesterone substitution protocol on the endometrial expression of estrogen-sensitive genes during the peri-implantation period. METHODS: Peripheral blood and endometrial biopsies were obtained from 13 infertile women both in a natural cycle (NC), on days 5 and 7 after ovulation (NC5, NC7), and in an artificial (substituted) cycle (AC), on days 5 and 7 of progesterone addition (AC5, AC7). Estrogen receptor-α (ERα) and progesterone receptor (PR) were assayed by immunohistochemistry. Matrix metalloproteinase-26 (MMP-26) mRNA and tissue inhibitor of metalloproteinase-4 (TIMP-4) mRNA were semiquantitatively assessed in tissue sections using in situ hybridization (ISH) and quantified in tissue extracts using real-time PCR. RESULTS: Levels of both E₂ and progesterone were higher in the peripheral blood in AC than in NC. Also on day AC5, expressions of ERα, PR and MMP-26 mRNA (focally) were increased in the epithelium and TIMP-4 mRNA in the stroma. Expression levels of these genes dropped significantly between AC5 and AC7, but not between NC5 and NC7. Abnormally high levels in AC5 samples suggest overstimulation with E₂, and the rapid decrease between AC5 and AC7 suggests overstimulation with progesterone. CONCLUSIONS: In ACs, increased levels of E₂ in the blood exaggerate the endometrial expression of estrogen-sensitive genes, whereas higher levels of progesterone in the blood in the secretory phase exaggerate the drop in expression of these genes. Dramatic variations in the gene expression may not be optimal for the implantation process.

Key words: estrogen/implantation/mRNA/progesterone receptor/regulation

Introduction

Synchronization between maturation of the endometrium and developmental stage of the transferred embryo is pivotal for successful implantation in IVF programmes. The use of cryopreserved embryos together with endometrial preparation allows optimizing the time of embryo transfer. Transfer of frozen-thawed embryos can potentially be performed in natural cycles (NCs) with unprepared endometrium after spontaneous ovulation or light ovulation induction. However, this approach is often hampered by ovulation disturbances, irregularities of the menstrual cycle, complicated monitoring of ovulation and inconvenient timing of embryo transfer.

Artificial preparation of the endometrium by exogenous steroids brings advantages for the patients, e.g. a lower risk of cycle cancellation and pre-scheduled time of embryo transfer, and is furthermore beneficial for patients with menstrual irregularities or ovarian dysfunction. Some protocols employ pituitary down-regulation with GnRH agonists to avoid spontaneous ovulation before sequential administration of estrogens and progesterone. However, these patients may encounter hypo-estrogenic side effects before the steroid replacement is initiated, and these protocols are longer and more costly. Lelaidier et al. (1992) reported a protocol for artificial endometrial preparation with exogenous steroids without preceding down-regulation. Subsequently, various protocols for endometrial preparation using stable doses (Massai et al., 1993; Younis et al., 1996; Simon et al., 1999) or incremental doses (de Ziegler et al., 1991) of estradiol (E₂) to mimic NCs have been devised.

E₂ and progesterone exert their effects through specific nuclear receptors (Clark and Peck, 1979). E₂ induces the expression of its own receptor [estrogen receptor-α (ERα)], as well as the progesterone receptor (PR) (Milgrom and Baulieu, 1970; Jensen and DeSombre, 1972; Janne et al., 1975; Leavitt et al., 1977). Progesterone-activated PR mediates
down-regulation of ERα, which subsequently leads to decreased expression of estrogen-sensitive genes, e.g. PR. Thus, progesterone induces a decrease of both ERα and PR in glandular epithelial cells (GECs) (Milgrom et al., 1973; Tseng and Gurpide, 1975).

During the normal menstrual cycle, the level of ERα staining is maximal in the early proliferative phase in all cell types of the endometrium. It declines sharply in the early secretory phase in endometrial stromal cells (SCs) and in the mid secretory phase in epithelial cells and is low in all cell types in the late secretory phase (Press et al., 1984; Bergeron et al., 1988; Lessey et al., 1988; Snijders et al., 1992; Ben-Hur et al., 1995; Mertens et al., 2001; Pilka et al., 2004a,b). The other ER (ERβ) is much less expressed and has probably more specialized functions in the endometrium (Lecce et al., 2001).

Successful implantation depends on the co-operation between the invasive blastocyst and the receptive endometrium. The achieved high fertilization rates continue to contrast with the low implantation rates (Nikas et al., 1995). Most authors agree that this discrepancy mainly results from altered endometrial conditions, which may affect receptivity.

The process of implantation and formation of placental vili is associated with remodelling of endometrial tissue through degradation of extracellular matrix (ECM) by proteases (Bischof et al., 1995). Certain matrix metalloproteinases (MMPs) are involved in ECM degradation and tissue remodelling during implantation (Salamonson, 1999). The family of human MMPs includes at least 24 members, which are classified into eight distinct classes (Egeblad and Werb, 2002). Enzymatic activity of the MMPs is restricted by tissue inhibitors of metalloproteinases (TIMPs) by forming 1:1 enzyme-inhibitor complexes (Brew et al., 2000). So far, four mammalian TIMPs have been characterized: TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Welgus and Stricklin, 1983; Stetler-Stevenson et al., 1989; Apte et al., 1995; Greene et al., 1996).

MMP-26 (endométase or matrilysin-2) is a novel enzyme, which has been cloned from fetal, endometrial tumour and placental cDNA libraries (de Coignac et al., 2000; Park et al., 2000; Uria and Lopez-Otin, 2000; Marchenko et al., 2001). It is expressed in epithelial cells, normal as well as malignant, and in malignant tumours including endometrial carcinoma (Park et al., 2000; Tunuguntla et al., 2003; Pilka et al., 2004b). We recently reported that MMP-26 mRNA is localized in the epithelial cells of the human endometrium and is maximally expressed in the early secretory phase (Pilka et al., 2003).

Kinetic studies have demonstrated that TIMP-4 has high inhibitory affinity for MMP-26 (Liu et al., 1997; Zhang et al., 2002). We have recently shown that TIMP-4 is expressed in the endometrial stroma and that the cyclic expression of TIMP-4 is co-ordinated with that of MMP-26, suggesting similar regulatory mechanisms (Pilka et al., 2004a). The cyclic pattern suggests regulation by ERα. In fact, we have reported sequences in the promoter region of both genes, which can potentially function as estrogen response elements (Pilka et al., 2004a,b).

This study is part of a more extensive effort to highlight possible effects of a substitution protocol on endometrial parameters in the implantation period (Oborna et al., 2004). We compared the endometrial expression of the estrogen-regulated genes ERα, PR, MMP-26 and TIMP-4 in NCs and artificial (substituted) cycles (ACs).

### Materials and methods

#### Substitution and sampling protocols

Thirteen women in infertile couples were recruited from the IVF programme at the Palacky University Center for assisted reproduction between 2001 and 2003. All women gave their written consent, and the Institutional Review Board of the Palacky University approved the study.

All women had a history of infertility of more than 12 months, were less than 40 years old, had regular menstrual cycles with normal concentration of serum progesterone in the mid-luteal phase and had physiological basal serum levels of FSH and prolactin on day 3 of a previous cycle. The preceding infertility workup identified tubal factor (n = 5), immunologic factor (n = 2), history of endometriosis (n = 1), idiopathic (n = 3) and male factor (n = 8). Two consecutive menstrual cycles were included in the study.

In the NC, all subjects were monitored for urinary LH (LH; Simtech Biore Inc, New York, USA) daily from day 10 of the cycle. From day 11 on, vaginal ultrasound examination (Hewlett Packard, probe 7.5 MHz) together with assay of serum E2 and progesterone was performed with 2 days interval until the day of ovulation. Endometrial biopsies were obtained on days 5 and 7 after ovulation (NC5, NC7).

In the AC, oral hormonal substitution was initiated on the first day of the cycle and continued with escalating doses of E2-valerate (days 1–5: 2 mg/day, day 6–10: 4 mg/day, day 11–15: 6 mg/day). From day 11 on, serum levels of E2 and progesterone were measured with 2 days interval, as in NC. The endometrium was evaluated on day 15. If endometrial thickness was 8 mm, micronized progesterone (600 mg/day orally) was added, and the dose of E2-valerate was decreased to 4 mg/day. If endometrial thickness was <8 mm, substitution continued with the same dose of E2-valerate (6 mg/day), and the ultrasound evaluation of the endometrium was repeated with 2-day intervals until 8 mm thickness was reached.

Endometrial biopsies were obtained on days 5 and 7 of progesterone substitution (AC5, AC7).

#### Endometrial tissue sampling

Endometrial biopsies were obtained with patients under sedation on day 5 and 7 in both NC and AC by a single investigator (I.O.), using a Novak curette. To assure that samples were taken from different areas, we took day 5 biopsies from the right anterior side of the uterine cavity and day 7 biopsies from the left anterior side. Each sample was divided into two aliquots. One portion was fixed in methacarn for 24 h, embedded in paraffin and later used for steroid receptor evaluation. The other portion was immediately frozen on dry ice and later used for MMP-26 and TIMP-4 mRNA analyses.

#### Hormone assays

Serum levels of E2 were assayed throughout all cycles (RIA kit 1663; Immunotect Inc., Prague, Czech Republic). Reference levels were 0.37–1.65 nmol/l in the preovulatory phase and 0.18–0.92 nmol/l in the mid-luteal phase. The inter-assay variation was 4–8%.

Serum levels of progesterone were assayed in the latter part of each cycle (RIA kit 1188, Immunotect Inc.). Reference levels were 0.2–4.0 nmol/l in the follicular phase and 8–78 nmol/l in the luteal phase. The inter-assay variation was 8–10% in the follicular phase and 3–4% in the luteal phase.
Serum levels of sex hormone-binding globulin (SHBG) were assayed in all cycles (RIA kit 3532, Immunotech Inc.). Reference levels were 30–100 nmol/l throughout the cycle, and the inter-assay variation was 4–8%.

**RNA extraction**

One portion of each frozen sample was disintegrated with a microdis- membrator, weighed and extracted for total RNA using Trizol™ (Life Technologies, Sweden). Frozen samples were homogenized in 1 ml of Trizol per 50 mg tissue and centrifuged for 15 min at 4°C and 12 000 g. After incubation for 10 min at room temperature, 0.2 ml chloroform per 50 mg tissue was added to the supernatant. Samples were mixed by vortexing for 15 s and subsequently centrifuged for 15 min at 4°C and 12 000 g. The supernatant was mixed with isopropanol and salt solution (0.8 mol/l Na-citrate and 1.2 mol/l NaCl) 0.75 ml per 50 mg tissue and stored at −20°C for 60 min. After sedimentation, samples were centrifuged for 30 min at 4°C and 12 000 g. Supernatant was discarded and the pellet was dried with 75% ethanol, 1 ml per 50 mg tissue. RNA pellet was air-dried and diluted in Rnase-free water. Total RNA was estimated from the absorbance at 260/280 nm quotients. Each sample was run on a gel to exclude degradation of the RNA.

**Preparation and labelling of complementary RNA probes**

For the human MMP-26 mRNA, a probe corresponding to 500 NT (225–725) was used, Genbank accession number AF248646 (Park et al., 2000). For the human TIMP-4 mRNA, a probe corresponding to 440 NT (231–670) was used, Genbank accession number 003256 (Greene et al., 1996). DNA templates were generated by PCR amplification from the human MMP-26 and TIMP-4 cDNAs, using bipar- titic primers consisting of either a modified T7 RNA promoter and a downstream gene-specific sequence (antisense) or a modified T3 RNA promoter and an upstream gene-specific primer (sense). PCR reactions using 1 ng human MMP-26 or TIMP-4 cDNA, 1 μg primer, 200 μmol/l dNTPs, 3 mmol/l MgCl2, 10 mmol/l Tris (pH 8.3), 50 mmol/l KCl, 2.5 units Taq polymerase (Invitrogen, Carlsbad, CA, USA) were amplified at 95°C for 1 min, 62°C for 1 min and 72°C for 1 min. The reaction was repeated for 30 cycles with a final extension at 72°C for 10 min. Expected size of the transcripts was verified with agarose gel. DNA templates were purified using QIAquick Gel Extraction Kit 250 (Qiagen GmbH, Hilden, Germany). Complementar- ry RNA (cRNA) probes were transcribed from 5 ng of DNA template using 35SUTP (Amersham Biosciences, Little Chalfont, UK; 800 Ci/mmol, 1 × 106 cpm per 50–80 μl of probe) and either T3 or T7 RNA polymerase according to manufacturer’s instructions (Ambion MAXIscript, Austin, TX, USA) to generate sense and antisense probes, respectively.

**In situ hybridization**

The other portion of each frozen sample was processed for *in situ* hybridization (ISH). Cryostat sections 14 μm thick were collected on siliconized glass slides and subsequently stored at −80°C until used for ISH. Before hybridization, tissue sections were pretreated as described (Young, 1990). Sections were thawed directly in 4% formaldehyde in phosphate-buffered saline (PBS) and fixed for 5 min, rinsed twice in PBS and acetylated in 0.1 mol/l triethanolamine–HCl, 0.25% acetic anhydride (pH 8) for 10 min. Sections were then rinsed twice in ×2 saline-sodium citrate (SSC) and dehydrated in ethanol 70% for 1 min, 80% for 1 min, 95% for 2 min, 100% for 1 min and 95% for 1 min, before air-drying. Hybridization histochemistry was performed as described by Cox et al. (1984) and Whitfield et al. (1990).

**Real-time PCR**

Aliquots of RNA (0.5 μg) from each sample were reverse transcribed using superscript II according to the manufacturer’s manual (Invitro- gen) and diluted into 100 μl with water. Subsequently, the cDNA (2 μl) was amplified by real-time PCR with TaqMan universal PCR mastermix (Applied Biosystem, Foster City, CA, USA). MMP-26 (Hs00222320_m1) and TIMP-4 (Hs00162784_m1) Assay-on-Demand kits from Applied Biosysytem were used. β-Actin was used as a housekeeping gene to normalize the results. Primers for β-actin were designed using the Primer Express software (Applied Biosystem), and 200 μmol/l of each primer and 1.25 pmol/l of probe were used. The primers for β-actin were β-actin-FW: 5’CTGGCTGCTGACCGAGG-3’ and β-actin-RW: 5’GAAAGTCTCATAACATGATCTGGGT-3’. The probe was β-actin-TM: 6FAM5′-CCCTGAACCCCAAG-GCCAACC-3’TAMRA. Each sample was analysed in duplicate using ABl prism 7000 (Applied Biosystem). The PCR amplification was related to a standard curve.

**Immunohistochemistry**

Methacarn-fixed, paraffin-embedded sections were treated with 10 mmol/l citrate buffer (pH 6.0) in a microwave oven at 550 W for 17 min for antigen retrieval (Shi et al., 1991). The hormone receptors were detected using monoclonal antibodies for ERα (ER1D5, Immuno- notech Inc.) and PR (PR1A6, Immunotech Inc.). Both antibodies were diluted to 1:50. The antibody for PR detected both isoforms of PR by western blot but has been reported to detect only PR-A by immunohis- tochemistry (Mote et al., 2001). The detection system included a biotinylated goat anti-mouse IgG antibody diluted to 1:50 (Immu- notech Inc.), streptavidin–AP conjugate and nitro blue tetrazolium (NBT)/5-bromo-α-chloro-3-indolyl phosphate (BCIP) with added levamisole as a substrate (Roche Diagnostics GmbH, Penzberg,
100% and 1 is a correction for optical density. The results ranged from the percentage of stained nuclei for each intensity varying from 0 to with a value of 1, 2 or 3 (weak, moderate or strong, respectively), 1986). Staining for ER each specimen. Histological score (HSCORE) was calculated using the analysis (ACC 4.0 software) was used to evaluate immunostaining of nuclei in cells that were randomly selected from three fields within each specimen. Histological score (HSCORE) was calculated using the equation: 

\[ \text{HSCORE} = \sum (i + 1) \]

where \( i \) is the intensity of staining with a value of 1, 2 or 3 (weak, moderate or strong, respectively), \( P_i \) is the percentage of stained nuclei for each intensity varying from 0 to 100% and 1 is a correction for optical density. The results ranged from 0 for no staining to 4 for maximal staining (Budwit-Novotny et al., 1986). Staining for ER\( \alpha \) and PR was estimated separately in GECs, luminal epithelial cells (LECs) and SCs.

**Statistical methods**

Results are presented as median and percentiles. The significance of differences between groups was evaluated with Wilcoxon test for matched-pairs, and \( P < 0.05 \) was considered statistically significant.

**Results**

Conventional histological evaluation of all endometrial samples was performed by a single observer (V.L.) according to Noyes criteria (Noyes et al., 1950). In NCs, 10 of 13 biopsies taken on day O+5 (day 19) were estimated in phase. The remaining three were 1-day delayed (day 18). The second biopsies were taken on day O+7 (day 21). Nine of 13 were in phase, three were delayed (day 20) and one was advanced to day 22. Similarly, in ACs, in seven of 13 biopsies taken on day P+5 (day 19), two were delayed (day 18) and four were advanced to day 20. The second biopsies taken on day P+7 (day 21) corresponded all but one to day 21; in one case, 1-day delay of histological dating was reported (day 20).

Immunostaining for ER\( \alpha \) in both LECs and GECs was stronger in AC5 than in NC5 (Figure 1). Also, it was stronger in AC5 than in AC7 but was not different between NC5 and NC7. In contrast, SC expression of ER\( \alpha \) was not affected by the substitution. Plasma levels of E2 were generally higher in AC than in NC.

Immunostaining for PR in both LECs and GECs tended to be stronger in AC5 than in NC5 and was significantly stronger in AC5 than in AC7 (Figure 2). Furthermore, immunostaining for PR in GECs tended to be more intense in NC5 than in NC7. Staining for PR in SCs did not vary significantly. Plasma levels of progesterone were generally higher in AC than in NC.

MMP-26 mRNA was evenly distributed in all GECs and LECs but was absent in the stroma (Figure 3). Sections hybridized with the sense (control) probe had no signal. Semiquantitative assessment of the hybridization signal showed that the intensity was higher in AC5 than in both NC5 and AC7 (Figure 4). Also, the signal was stronger in NC7 than in AC7. MMP-26 mRNA was assayed with real-time PCR and values normalized to the corresponding \( \beta \)-actin mRNA level. The MMP-26 probes for real-time PCR and ISH recognized non-overlapping sequences. The amount of MMP-26 mRNA was greater in samples from AC5 than in those from AC7 (Figure 4).

TIPM-4 mRNA was evenly distributed within the stroma in all endometrial samples (Figure 5). No signal was detected in the epithelial cells. Sections hybridized with the sense probe had no signal. The TIPM-4 mRNA signal was more intense in AC5 than in NC5 (Figure 6). Also, it was stronger in AC5 than in AC7. Real-time PCR values for TIPM-4 mRNA were normalized to \( \beta \)-actin mRNA. The probes for real-time PCR and ISH recognized non-overlapping sequences. The amount of TIPM-4 mRNA was higher in AC5 than in AC7 (Figure 6).

**Discussion**

This study used mRNA for MMP-26 and TIPM-4 in endometrial tissue to assay for the effects of steroid hormones in a substitution protocol. We used quantification with real-time PCR as well as semiquantitative estimation of the ISH signals. Results of the two methods showed the same trend. However, in several cases variations in mRNA level were significant only for the ISH signal and not for the real-time PCR data. Although real-time PCR is a quantitative method, variations in a limited cell population may not be detected, because RNA for analysis is extracted from the whole tissue. This results in ‘dilution’ of the signal, and discrete variations may be lost. The ISH signal, on the contrary, is evaluated only within the cellular compartment where the mRNA is expressed.

Various hormonal substitution protocols are reportedly successful in supporting implantation (Lutjen et al., 1984; Navot et al., 1986; Rosenwaks, 1987; Droesch et al., 1988).

Histological studies of the endometrium in substituted cycles have revealed minor delay of maturation in the midluteal phase (Lutjen et al., 1984; Rosenwaks, 1987; Droesch et al., 1988; Navot et al., 1989; Steingold et al., 1989; de Ziegler et al., 1992). This difference was, however, transient because no delay of maturation was found after 10 days of progesterone substitution. The discrepancy was suggested to be due to the discrete elevation of plasma progesterone that occurs before ovulation in NC but is not mimicked in AC. Hormone replacement regimens mimicking the estrogen and progesterone plasma levels in NCs resulted in phase patterns, which were consistent with normal endometrial morphology (Sauer et al., 1991; Paulson et al., 1997). Standard histological endometrial dating was performed in our material according to Noyes et al. (1950). The maximum dyssynchrony in endometrial secretory changes seen in our study was never more than \( \pm 1 \) day in both NC and AC (Oborna et al., 2004). According to Jordan et al. (1994) such differences are not considered to be out of phase. However, lack of morphological differences in endometrial maturation does not exclude biochemical differences.

This report compares endometrial expression of estrogen-sensitive genes in AC and NC. Each patient was sampled twice in NC and twice in AC on comparable days of the cycle. We observed on day 5 that epithelial immunostaining was more intense for ER\( \alpha \) and tended to be more intense for PR in AC as
compared with NC. This presumably results from the higher plasma levels of E₂ in AC. Between days 5 and 7, the epithelial staining for both ERα and PR dropped significantly in AC, and PR, but not ERα, levels tended to decline in NC. This is partly in contrast to the earlier studies characterized by the disappearance of both ER and PR staining during the mid-luteal period of the normal menstrual cycle (Press et al., 1984; Garcia et al., 1988; Press et al., 1988). Press et al. (1988) observed PR immunostaining to decrease sharply already at postovulation day 4, whereas Lessey et al. (1988) reported dyssynchronous fluctuations of PR content during the secretory phase. The discrepancies between the studies might be explained by different timing of biopsies or different antibodies used. In fact, it is possible that the major drop of ERα expression in our study has already occurred on day NC5, and we observe between NC5 and NC7 decreasing expression in genes, which are secondarily regulated by ER. Massai et al. (1993), who studied patients with oral administration of E₂ and vaginal administration of progesterone, found staining for PR to be strong in glandular cells as well as SCs on day 5 of progesterone substitution, whereas PR staining had disappeared in the glands in three of six biopsies on day 7. Even though the timing of our biopsies coincides with the normal drop of PR expression, a significant drop was not found between NC5 and NC7 in this study.
Despite lower doses of exogenous steroids in their study, Massai et al. also found a drop in epithelial expression of both ERα and PR between days 5 and 7. Recently, two PR isoforms have been identified, namely PR-A and PR-B (Kastner et al., 1990b). Because PR-A and PR-B are proposed to have different functions, and because they exist at different relative levels in various tissues, the ratio of PR-A/PR-B is likely to affect the response to progesterone (Kastner et al., 1990a; Tung et al., 1993; Vegeto et al., 1993; Wen et al., 1994). During the menstrual cycle, PR-A and PR-B are similarly expressed in the proliferative phase, PR-A predominantly expressed in the early secretory phase and PR-B predominantly expressed in the mid secretory phase (Mote et al., 1999; Arnett-Mansfield et al., 2004). Moreover, Mote et al. (2001) showed that most antibodies to PR fail to detect PR-B in tissue sections by immunohistochemical techniques. As a result, PR expression may be underestimated in tissues where PRB is predominant. Differences between immunohistochemical studies of PR expression may thus, at least partly, be explained by the use of different PR antibodies.

To further evaluate the functional aspect of ERα, we compared expression of the estrogen-sensitive genes MMP-26 in the epithelium and TIMP-4 in the stroma between AC and NC.
Our observation on the cyclic variation of MMP-26 gene expression in the endometrium suggested regulation by ERα, and in fact, we found a potential estrogen response element (ERE) in the promoter region of the gene (Pilka et al., 2004b). This is in agreement with an observation by Chegini et al. (2003), who also found highest levels of endometrial MMP-26 at mid-cycle of women with normal menstrual cycles. However, their finding of MMP-26 expression in epithelial, stromal and smooth muscle cells is in contrast with the epithelial distribution observed by both Isaka and ourselves (Isaka et al., 2003; Pilka et al., 2003; Pilka et al., 2004b). Progesterone is a potent repressor of MMP expression both in vivo and in vitro. Thus, most MMPs are expressed when the concentration of progesterone is low, i.e. in the proliferative and menstrual phases. In contrast, expression of most MMPs decreases in the secretory phase, when the concentration of progesterone is high (Rodgers et al., 1993; Hampton and Salamonsen, 1994; Rodgers et al., 1994; Marbaix et al., 1995; Kokorine et al., 1996; Goffin et al., 2003). The unique expression pattern of MMP-26, different to all other known endometrial MMPs, suggests also different regulatory mechanisms.

Recently, Li et al. (2004) confirmed presence of a functional ERE sequence in the MMP-26 promoter. Marchenko et al. (2002) characterized the promoter region of the MMP-26 gene, exhibiting a few transcription factor-binding motifs. Only AP-1 and Tcf-4 sites appeared to be functional. They suggest that MMP-26 gene expression regulation by AP-1 may involve Ras and Jun families of proteins. This is in agreement with our observation on cyclic variation of endometrial c-jun expression (Pilka et al., 2003). On the contrary, stimulation of the MMP-26 promoter by β-catenin involves the Tcf-4 site and the Wnt signal transduction pathway (Marchenko et al., 2004). Regulation of MMP-26 gene thus seems to be complex.

The amount of stromal TIMP-4 mRNA increases from the early proliferative phase to peak expression in the early secretory phase and then gradually decreases to the menstrual phase in normal cycle (Pilka et al., 2004a). Thus, the cyclic expression pattern of TIMP-4 is similar both to that of MMP-26 and ERα and, taken together with our finding of a potential ERE sequence up-stream of the transcribed region, strongly suggests that the TIMP-4 gene is regulated by ERα (Pilka et al., 2004a). A previous report by Chegini et al. (2003) on endometrial TIMP-4 agrees with our results on the cyclic expression pattern. However, as for MMP-26, this group again reports distribution in stromal, epithelial and smooth muscle cells. The discrepancy may relate to the fact that they rely on immunohistochemistry as the only method.

In this study, we found that the content of TIMP-4 mRNA in the stroma was focally higher on day AC5 than on both day NC5 and day AC7. This result, which is similar to that of MMP-26, ERα and PR in the epithelium, is consistent with regulation by ERα and suggests overstimulation during both estrogen and progesterone substitution. However, the TIMP-4 mRNA variation is in contrast to the unchanged staining intensity of ERα in SCs. The result suggests that regulatory mechanisms, other than exclusively ERα, are involved in the control of TIMP-4 mRNA levels. Progesterone has been shown to be a potent regulator of most endometrial MMPs. However, no effect of progesterone withdrawal was observed on TIMP-1.

In NC, epithelial content of MMP-26 mRNA rapidly decreases between highest levels in the early secretory phase and zero levels in the late secretory phases (Pilka et al., 2003). However, in this study we found no difference in epithelial expression of MMP-26 mRNA between NC5 and NC7, presumably due to the short interval between samplings. In contrast, we found a significant decrease between AC5 and AC7. Also, the mRNA signal was focally higher in AC5 than in NC5. These observations in NC and AC correlate with the immunostaining intensity for ERα in GECs, the main cellular compartment of MMP-26 mRNA expression. Apparently, this substitution protocol overstimulates expression of the MMP-26 gene during estrogen substitution and causes a rapid drop during progesterone substitution.
MMP-26 and TIMP-4 in artificial cycles

TIMP-2 or TIMP-3 protein or mRNA production (Salamonsen et al., 1997). Similarly, our results do not support possible regulation of endometrial TIMP-4 mRNA expression by progesterone. Transcriptional regulation of TIMP-4 by interleukin-13 has been reported in lung tissue (Ma et al., 2004), and two independent groups have characterized the promoter region of the TIMP-4 gene and demonstrated Sp1 and Inr-like elements with functional importance for basal gene expression (Rahkonen et al., 2002; Young et al., 2002).

However, TIMP-4 has a predominant cardiac expression (Greene et al., 1996), and recently a discrepancy between mRNA and protein levels was reported in myocardial tissue. Left ventricular TIMP-4 protein levels, but not mRNA levels, were significantly lower in spontaneously hypertensive heart failure in rats (Li et al., 2000). In contrast, left ventricular TIMP-4 protein was up-regulated in patients with aortic stenosis, whereas TIMP-4 mRNA was unchanged (Fielitz et al., 2004). The finding of a binding site for the translational controller heterogeneous nuclear ribonucleic protein E, which is present in the 3′ untranslated region of TIMP-4, suggests that translational control is involved in the regulation of TIMP-4 protein levels (Li et al., 2000; Reimann et al., 2002; Fielitz et al., 2004).

The significance of the MMP-26 and TIMP-4 genes for reproductive processes is still unknown, but the effects seen on these genes is likely to be representative of estrogen-responsive genes in the endometrium. Substitution protocols with overstimulation may adversely influence the implantation process.

Acknowledgement
This project was supported by grants from the Swedish Cancer Fund; the Swedish Medical Research Council (12660, 14358, 14187); the Medical faculty at Lund University; the Lund University Hospital fund for cancer research; Nilsson, Crafoord and Kamprad foundations; the King Gustaf V and Queen Victoria Foundation; and IGA NH 6611/3, Ministry of Health, Czech Republic.
Figure 6. Semi-quantitative evaluation of the TIMP-4 mRNA in situ hybridization signal (left panel) and quantification of TIMP-4 mRNA/β-actin mRNA by real-time PCR (right panel). The signal was stronger in AC5 than in NC5 ($P = 0.008$). Also, it was higher in AC5 than in AC7 ($P = 0.003$). The amount of TIMP-4 mRNA assayed with real-time PCR was higher in AC5 than in AC7 ($P = 0.05$). Results are presented as box plots showing 10th, 25th, 50th (median), 75th, and 90th percentiles.

References


Submitted on September 19, 2005; resubmitted on January 2, 2006, March 10, 2006, April 21, 2006; accepted on April 28, 2006.