Pro-protein convertases (PCs) other than PC6 are not tightly regulated for implantation in the human endometrium

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Abstract

Pro-protein convertases (PCs) are a family of serine proteases (furin, PC1/3, PC2, PACE4, PC4, PC5/6, PC7/8) responsible for post-translational processing and activation of inactive precursors of many regulatory proteins. Endometrial PC6 is critical for implantation in mice and for decidualization of human endometrial stromal cells (ESCs). This study investigated the endometrial expression of other PCs during the menstrual cycle and early pregnancy to elucidate potential redundancies. Furin, PC4, PACE4, and PC7 along with PC6 transcripts were detected in total endometrial RNA, whereas PC1 and PC2 transcription levels were negligible. Quantitative RT-PCR demonstrated highest levels of furin mRNA during menstruation and lowest levels during the proliferative phase. Furin protein was immunolocalized in endometrial luminal and glandular epithelia, stromal fibroblasts, endothelia, and leukocytes. PACE4 and PC7 proteins were also immunodetected in endometrial stroma and glands. Total furin, PC7, and PACE4 proteins were constitutive in both stromal and glandular compartments throughout the cycle and during first trimester pregnancy. Furthermore, Furin and PC7 transcription was unaltered during decidualization of ESCs *in vitro* in contrast to PC6 which is significantly up-regulated during decidualization. Thus, whereas PC6 is tightly regulated during endometrial preparation for implantation, furin, PACE4, and PC7 are constitutively expressed in human endometrium, but must be considered if PC6 is to be targeted for manipulation of fertility.

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Introduction

The human endometrium undergoes cyclic growth, remodeling, and functional changes that are hormonally synchronized with the maturation and release of the ovum. During every menstrual cycle, the endometrium repairs and proliferates after menstruation, differentiates in preparation for pregnancy, and breaks down and is shed during menstruation in the absence of an implanting conceptus. Based on both morphology and gene expression (Noyes *et al.* 1975, Ponnampalam *et al.* 2006), the developmental state of the endometrium can be described as menstrual (M), proliferative (P), early, mid- or late secretory (ES, MS or LS). Transformation of stromal fibroblasts into decidualized cells is initiated during the MS to LS phase, particularly around spiral arteries and close to the luminal epithelium (Loke & King 1995). This progresses into the first trimester of pregnancy, when maximal decidualization is achieved.

We recently demonstrated that pro-protein convertase 5/6 (PC6) mRNA and protein are induced specifically in decidualizing cells of mice and humans, and that specific inhibition of this induction at a very early stage

using anti-PC6 morpholino oligonucleotides *in vivo* blocks decidualization and prevents implantation in the mouse (Nie *et al.* 2003, 2005a). In addition, blocking of PC6 production during decidualization of human endometrial stromal cells (ESCs) *in vitro*, significantly reduces decidualization (Okada *et al.* 2005). PC6 also cleaves the human immunodeficiency virus (HIV) envelope protein as a requirement for HIV infectivity (Taylor *et al.* 2003). Based on these characteristics, we suggested that PC6 could be used as a novel target in female contraception with an additional protective function against HIV (Nie *et al.* 2005a,b).

PC6 belongs to the PC family of serine proteases. There are six additional mammalian PCs (furin, PC1/3, PC2, PC4, PACE4, PC7/8) that are structurally and functionally highly homologous and like PC6 cleave polypeptides at basic residues within the general motif of (K/R)-(X)*n*-(K/R; Seidah & Chretien 1999). Thus, we aimed to determine whether PCs other than PC6 are expressed in the human endometrium during the menstrual cycle and early pregnancy, and to establish whether they are also key regulators for stromal cell decidualization.

PC1 and PC2 are thought to be restricted to neuronal and endocrine tissues, and PC4 appeared to be testis specific (Seidah & Chretien 1999, Bergeron *et al.* 2000), but was recently demonstrated in human first and third trimester placenta (Qiu *et al.* 2005). In contrast to these, distinctly expressed PCs, furin, PACE4, and PC7 are ubiquitously expressed (Bergeron *et al.* 2000). Furin is the best characterized member of the PC family (Denault & Leduc 1996). The furin mRNA gives rise to an inactive pro-peptide that undergoes proteolytic maturation into a membrane-bound protein localized mainly in the trans-Golgi network (Denault & Leduc 1996, Nakayama 1997, Bergeron *et al.* 2000). A soluble isoform can also be produced by cleavage of the transmembrane and cytosolic region (Paleyanda *et al.* 1997). Similarly, PACE4 and PC7 have membrane bound (MB) and soluble isoforms, which are, however, generated through transcriptional variation (Zhong *et al.* 1996, Munzer *et al.* 1997).

The putative and confirmed substrates of PCs include precursors of adhesion molecules, growth factors, cytokines, their receptors, and proteolytic enzymes (Nakayama 1997, Seidah & Chretien 1999), many of which are implicated in implantation and other endometrial physiological functions. However, the *in vivo* cleavage of any substrate depends on the relative activity levels of all PCs that can cleave this substrate within the microenvironment (Nakayama 1997).

Materials and Methods

Tissue collection and processing

Human endometrial tissues were obtained as previously described during the menstrual (M), proliferative (P), ES, MS, and LS phases of the menstrual cycle (Jones *et al.* 2004). Early pregnant (6–8 weeks) decidua (D) was collected from women undergoing elective termination of pregnancy. Approval was obtained from the Human Ethics Committee at Monash Medical Centre, Melbourne, and the women gave informed consent. Stage of the cycle was confirmed by histological assessment according to the criteria of Noyes *et al.* (1975). Samples were fixed or frozen and processed as described (Jones *et al.* 2004).

Human ESCs were collected and cultured in a previous study (Okada *et al.* 2005). Briefly, ESCs were isolated from tissue by enzymatic digestion and filtration, cultured in DMEM-Ham's F12 medium supplemented with 10% charcoal-treated fetal calf serum and 1% antibiotics at 37 °C in a humidified atmosphere of 5% CO₂ in air. The culture medium was replaced 60 min after plating. Purity of ESCs was determined by morphology and immunohistochemical staining with markers specific to ESCs.

In vitro decidualization

ESCs were decidualized in a previous study (Okada *et al.* 2005). Briefly, ESCs were plated in six-well plates, cultured until confluent and treated with estrogen (10–8 mol/l) in the presence or absence of medroxy-progesterone acetate (10–7 mol/l). The culture media were changed every 3 days. Cells were lysed for RNA extraction at 3, 6, 9, and 12 days of treatment.

RNA extraction

Total RNA was extracted from endometrial samples of menstrual ($n=3$), proliferative ($n=5$), ES ($n=4$), MS ($n=5$), and LS ($n=3$) phase of the menstrual cycle. Samples were homogenized in Trizol reagent (Qiagen Sciences) according to the manufacturer's instructions. All samples were DNase (Ambion, Austin, TX, USA) treated and quantified by photospectrometry. Purity and integrity of RNA was verified by photospectrometry and agarose gel electrophoresis. Total RNA from cultured ESCs that were decidualized with progesterone *in vitro* over a period of 12 days (three experiments) was isolated and processed in a previous study (Okada *et al.* 2005).

Primers

Primers for all PCs were designed with Primer3 software (<http://bioinformatics.vg>; Table 1). PC6 and 18S primers were previously described (Okada *et al.* 2005).

RT and real time PCR

One microgram of total RNA (DNA-free) from endometrium or ESCs was reverse transcribed in a 20 µl reaction. Three microliter of RT product for one sample each of proliferative and MS phase were amplified for all PCs in a 50 µl reaction mixture by conventional PCR over 35 cycles. PCR for all PCs and controls in reverse transcripts of total endometrial RNA was conducted in one common run, except for furin, which was amplified at a higher annealing temperature. For conventional PCR of furin, PC4, PACE4, and PC7 reverse transcripts from RNA of cultured ESCs, all samples of treatment and control cells from days 3, 6, 9, and 12 of the treatment period were included within the same run. PCR products (11.3 µl) were separated by 2% agarose gel electrophoresis. All PCR products were confirmed by sequencing.

For quantitative PCR, triplicate RT products of all samples were pooled, and SYBR Green (Molecular Probes Inc., Eugene, OR, USA) real time PCR was conducted as previously described (Jones *et al.* 2004). All samples were diluted 1:3 or 1:5. Standards were serial dilutions of purified conventional PCR products. Three runs of real-time PCR were performed for every sample of total endometrial RNA and one run for each of the three *in vitro* decidualization experiments of ESCs.

Table 1 List of primers.

mRNA	Accession	Fragment size (location on the mRNA)	Forward primer, reverse primer	T _a (°C)
Furin	BC012181	163 bp (1722–1885)	ACA ACT ATG GGA CGC TGA CC TGG ACA CAG CTC TTC TGG TG	57
PC1	NM_000439	151 bp (1580–1731)	TGT TCA CAC ATG GGG AGA GA ACG AGG CTG CTT CAT ATG CT	55
PC2	BC005815	186 bp (483–669)	GAG AAG ACG CAG CCT ACA CC CCA TCA GCT TGC CCA GTA TT	55
PC4	NM_017573	189 bp (515–703)	TAC ACG CTG CTG CTC TAT GG CAG CCT TGT GTG GTT GAA GA	55
PACE4	M80482	239 bp (4076–4315)	GCC AAA CCT GTG TAG GCA TT CTG GGT TCT CTC CAG CTC AC	55
PC7	U40623	218 bp (613–831)	GCA CCC AAC TAT AGC CCT GA TCC ATG CTG TCT GTG AGA GG	55

T_a, annealing temperature.

Amplification was conducted at 95 °C for 10 s, annealing temperature (Table 1) for 10 s and 72 °C for 7 s over 25–30 cycles. All samples to be compared were included within the same run. At the end of each program, melting curve analysis was carried out.

Data for endometrial RNA were not normalized for the expression of a housekeeping gene due to the regulated expression of all known housekeeping genes examined in the endometrium (Jones *et al.* 2004). However, quantitative PCR data for RNA from ESCs were normalized against the expression of 18S RNA as this showed constant levels throughout the treatment period. Relative expression levels within each run of real time PCR were calculated for each sample based on the sample with the highest level being set 100%. For total endometrial RNA, relative expression levels were averaged for each sample over the three runs and the mean of these averages calculated for each phase of the cycle and first trimester decidua. For RNA from *in vitro* decidualized ESCs, relative expression levels were averaged for each day of treatment over the three decidualization experiments.

Antibodies

Commercial polyclonal antibodies for furin, PACE4, and PC7 were available for this study (Table 2). The affinity purified antibodies to detect PC7 (sc-22903) and PACE4 (sc-22898) were commercially generated using peptides that are 12–20 amino acids long mapping between amino acids 680–730 of human PC7 (UniProt accession Q16549) and 850–900 of human PACE4 (UniProt accession P29122). BLAST alignment of the immunogen of PC7 and PACE4 did not reveal any significant alignments with other members of the PC family (Santa Cruz Biotechnology, Santa Cruz, CA, USA, personal communication). Two antibodies for furin were used, directed either against the MB form or MB and soluble (MB&S) forms. The antibody directed against MB&S furin (sc-20801) was raised against a large region of furin (amino acids 575–794). A protein BLAST of the

immunogen returns alignment scores of 50–80% for PC4 and 40–50% for PC6. However, crossreactivity with these PCs was excluded by western blotting. Alignment scores for all other PCs are below 40% and therefore negligible. The antibody directed against MB furin (ALX-210-134) was raised against a synthetic peptide corresponding to amino acids 781–793 (GERTA-FIKDQSAL) of C-terminal mouse furin convertase (NCBI accession P23188), which does not return any significant alignment scores with other PC family members.

Immunoprecipitation and western blotting

Immunoprecipitation followed by western blotting of a menstrual phase endometrial protein extract was conducted to confirm validity of furin antibodies (Table 2). The endometrial sample (80 mg) was lysed in 1 ml radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris–HCL, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate, 1% NP-40, pH 7.5) with 10 µl/ml of protease inhibitor cocktail VII (Calbiochem, San Diego, CA, USA) and 0.3 µg/ml phenyl methylsulfonyl fluoride. 500 µl of pre-cleared extract was precipitated with 2 (sc-20801) or 10 µg (ALX-210-134) of primary antibody overnight at 4 °C, followed by binding to protein A/G conjugated agarose beads (20 µl, Santa Cruz Biotechnology) for 4 h at 4 °C. Beads were washed in RIPA buffer and boiled in 20 µl reducing sample buffer. Standard 8% SDS-PAGE of

Table 2 Polyclonal antibodies used in this study.

Target	Detected isoform	Host species	Catalogue number	Company
Furin	Membrane bound (MB)	Rabbit	ALX-2101-34	Alexis ^a
Furin	All isoforms (MB&S)	Rabbit	sc-20801	Santa Cruz ^b
PACE4	All isoforms	Goat	sc-22898	Santa Cruz
PC7	All isoforms	Goat	sc-22903	Santa Cruz

^aAlexis Biochemicals, Lausen, Switzerland. ^bSanta Cruz Biotechnology, Santa Cruz, CA, USA.

10–15 µl sample per well was followed by transfer onto a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, GE Healthcare). The membrane was blocked overnight in 5% skim milk in Tris-base buffer containing 0.05% Tween-20 (TTBS) at 4 °C and incubated for 1 h in primary antibody at room temperature (sc-20801 at 0.4 µg/ml; ALX210134 at 40 and 10 µg/ml). Membranes were then incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit immunoglobulin G (IgG; 1:20,000, DAKO, Botany, NSW, Australia) and developed by chemiluminescence (ECL Plus system, Amersham). Membranes stripped and incubated in normal rabbit IgG (DAKO) at 20 µg/ml served as negative controls.

Immunohistochemistry

Samples for furin immunohistochemistry were from menstrual ($n=3$ MB/ $n=4$ MB&S), proliferative ($n=4$ MB/ $n=7$ MB&S), ES ($n=3$ MB/ $n=6$ MB&S), MS ($n=4$ MB/ $n=6$ MB&S) and LS ($n=3$ MB/ $n=5$ MB&S) phases of the cycle, and from first trimester decidua ($n=4$ MB/ $n=4$ MB&S). Samples for PC7 and PACE4 immunohistochemistry were from each phase of the cycle ($n=1$ – 4), and from first trimester decidua ($n=2$).

Sections (5 µm, 2 µm only for serial CD45 staining) were de-waxed and re-hydrated. Heat induced epitope retrieval (10 mM citrate buffer, pH 6.0, 10 min) was followed by permeability enhancement and quenching for endogenous peroxidase and biotin/streptavidin-binding activity. PC7 and PACE4 antibodies and non-immune goat IgGs (Jackson ImmunoResearch, West Grove, PA, USA) were pre-incubated in blocking agent, consisting of casein (10%), normal horse serum (20%), fetal calf serum (6%), normal human serum (10%), and BSA (BSA, 1%) in salt enriched TTBS, for 2 h at room temperature. As a second negative control, 2 µg/ml PACE4 antibody was incubated with 10 µg/ml blocking peptide (sc22898P, Santa Cruz Biotechnology) in the same blocking agent, but without BSA, over the same period. BSA was omitted from the blocking agent used for the blocking peptide control to achieve equal

total protein/peptide concentrations for blocking agents used for treatments and controls. Tissues for all immunohistochemistry were incubated in blocking agent for 1 h at room temperature. The blocking agents for furin immunohistochemistry consisted of normal goat serum (10%), cold water fish gelatine (0.01%, for ALX-210-134) or fetal calf serum (6%, for sc-20801) and BSA (0.5%) in salt-enriched TTBS. Blocking was followed by incubation in 1.5 µg/ml (furin) or 2 µg/ml (PC7, PACE4) primary antibody or non-immune control IgG (DAKO) at 4 °C overnight. Secondary antibody incubation was in 2–4 µg/ml biotinylated goat anti-rabbit (furin) or 2 µg/ml biotinylated horse-anti-goat (PC7, PACE4) IgG (Vector Laboratories, Burlingame, CA, USA) for 30 min. Diluents were 0.5% BSA in salt-enriched TTBS, blocking agent (furin) or 2% normal human serum in salt-enriched TTBS (PC7, PACE4) respectively. Signals were amplified with Strep-TABC/HRP (DAKO) and visualized with diaminobenzidine (DAKO). Sections were counterstained with Harris hematoxylin, dehydrated and mounted with DPX.

Staining was assessed by H-scoring (Lessey *et al.* 1988) within each compartment (stromal fibroblast, glands), which considers the proportion of cells that are staining strongly, moderately, faintly, or lack staining. We also estimated the percentage of cells that stained at any intensity for each compartment. Some furin-stained sections were subsequently immunostained (double stained) for leukocyte common antigen (CD45). Sections (2 µm serial) that were cut immediately adjacent to sections that were stained for PACE4 were also stained for CD45. Excess peroxidase was quenched and sections were blocked in a cocktail of normal horse serum (10%), fetal calf serum (6%), and BSA (0.5%) in TTBS followed by incubation in primary antibody against CD45 (1:50, DAKO) at 4 °C overnight. Detection was as described above (2 µg/ml biotinylated horse-anti-mouse IgG) and visualized with DAB that was enhanced in the case of double staining (0.05% nickel ammonium sulfate, 0.05% cobalt chloride) to produce a dark blue-grey precipitate. Sections of first trimester decidua in close proximity to sections stained for furin and processed in

Figure 1 Pro-protein convertase amplicons of reverse transcripts from proliferative (P) and mid-secretory (S) phase endometrium. (A) Furin, PACE4, PC4, PC6, and PC7 mRNA are all expressed in the human endometrium. (B) In contrast, the levels of PC1 and PC2 transcripts are negligible in both P and S endometrial tissues but are present in mouse brain (+ve) controls. Reference to the housekeeping gene (18S) is shown in A and B. All samples were separated on the same gel.

the same manner were blocked as for CD45 and stained for cytokeratin with antibody (Becton Dickinson, North Ryde, NSW, Australia) diluted 1:4 in blocking agent at 4 °C overnight.

Figure 2 Relative furin and PC7 mRNA levels (mean $\pm$ S.E.M.). (A) Relative furin mRNA levels at the menstrual (M, $n=3$), proliferative (P, $n=5$), early secretory (ES, $n=4$), mid-secretory (MS, $n=5$), and late secretory (LS, $n=3$) phases of the cycle, and during first trimester pregnancy (D, $n=4$) in total endometrial RNA. Expression levels were higher during menstruation ($P<0.05$) compared with any other stage of the cycle and lower during the proliferative phase compared with the menstrual and early secretory phases ($P<0.05$). Relative furin (B) and PC7 (C) mRNA levels (normalized to 18S) during decidualization of stromal fibroblasts *in vitro* (mean of 3 separate experiments). No significant differences were detected between decidualizing treatment (■) and non-decidualized control (□) at days 3, 6, 9, and 12 of treatment. * $P<0.05$.

Statistical analysis

Statistical analysis was performed by One-way ANOVA and Tukey's HSD *post hoc* test using Prism 2.01 (GraphPad, San Diego, CA, USA) software.

Results

Most PCs are transcribed in human endometrium

Transcripts of furin, PC4, PACE4, PC6, and PC7 were detected by conventional RT-PCR in total endometrial RNA (Fig. 1). Although furin, PC4 and PACE4 mRNA could not be demonstrated in the sample of proliferative phase endometrium (P), their expression was shown in the MS phase sample (MS). Expression levels of PC4 and PACE4 mRNA were apparently lower than those of furin, PC6, and PC7. No significant signal could be achieved in the case of PC1 and PC2 mRNA. Validity of primers and PCR conditions for these PCs was confirmed on total mouse brain RNA (bottom panel, Fig. 1).

Furin and PC7 transcription is not up-regulated with decidualization

In total endometrial RNA, furin mRNA was detected throughout the menstrual cycle and in first trimester decidua (Fig. 2A). Levels were higher during the menstrual phase ($P<0.05$) than during any other phase of the cycle or early pregnancy, and lower ($P<0.05$) during the proliferative phase than during the ES phase.

Total RNA from ESCs decidualized *in vitro* contained furin and PC7 transcripts as demonstrated by conventional PCR, whereas PACE4 and PC4 transcripts were of too low abundance to allow for quantification in both treatment and control cells throughout the culture period (data not shown). However, quantitative PCR did not reveal any significant alterations of furin and PC7 mRNA levels with decidualization of ESCs *in vitro* (Fig. 2B and C).

Figure 3 Western blot analysis of furin in immunoprecipitates of an endometrial extract. (a) precipitated and detected with antibody against both furin isoforms (sc-20801), (b) precipitated with sc-20801, detected with antibody against the membrane-bound isoform of furin (ALX-210-134), (c) precipitated with sc-20801, detected with non-immune rabbit IgG, (d) precipitated and detected with ALX-210-134, and (e) precipitated with ALX-210-134 and detected with non-immune rabbit IgG. ns, non-specific background.

Immunoprecipitation and western blotting confirm furin antibody specificity

Depending on the extent of post-translational modification of furin protein, three different isoforms of furin can exist: latent membrane-bound (100 kDa), active membrane-bound (94 kDa), and soluble furin (80 kDa; Nakayama 1997). Following precipitation with the antibody against MB&S furin (sc-20801) and detecting with either the same antibody (Fig. 3 lane a) or the antibody against MB furin (ALX-210-134, Fig. 3 lane b), bands at ~100 and 96 kDa were evident. The band representing the soluble isoform was masked by a non-specific band of the same size, as evident by the appearance of the same band when the detection antibody was replaced with non-immune rabbit IgG (Fig. 3 lane c). Thus, the presence of soluble furin could not be shown. When precipitating and detecting with the antibody against MB furin only, 100 and 96 kDa bands

were apparent (Fig. 3 lane d); replacement of detection antibody with non-immune IgG also showed a non-specific band at 80 kDa (Fig. 3 lane e). Thus, both antibodies precipitate and detect latent and active furin.

Furin protein is immunolocalized to different endometrial cell types

MB furin was detected in endometrial glandular and luminal epithelial cells in all phases and in some stromal fibroblasts and decidualized cells (Fig. 4A–F). The staining pattern for MB&S furin was similar (Fig. 4G–K, menstrual phase similar to 4E). However, MB&S furin was consistently detected in stromal fibroblasts during the MS phase of the cycle (Fig. 4I), when staining for MB furin alone was very low (Fig. 4C). Neutrophils and endothelia of endometrial blood vessels (but not vascular smooth muscle) also

Figure 4 Representative photomicrographs of endometrial sections immunostained for furin. (A–F): Staining for membrane-bound (MB) furin, in proliferative (A), early secretory (B), mid-secretory (C), late secretory (D), menstrual phase tissue (E), and decidua of first trimester pregnancy (F, inset: negative control). (G–K): Staining for total furin (membrane-bound and soluble, MB&S) on samples of proliferative (G), early secretory (H), mid-secretory (I), late secretory phase tissue (J), and decidua of first trimester pregnancy (K, inset: negative control). (L): Double stain for MB&S furin (brown) and leukocyte common antigen (CD45, dark grey-blue) on a late secretory phase sample. Arrow heads: decidualized stromal cells, arrows: endometrial glands. Scale bars = 50 µm.

stained for furin using both antibodies. Well-decidualized stromal fibroblast during the LS phase and first trimester decidualization always stained strongly using both antibodies (Fig. 4F and K). All negative controls were clear of any stain (insets).

A small proportion of the furin-stained cells in the stromal compartment of both cycling endometrium and first trimester decidua were leukocytes as evident from double staining against MB&S furin and CD45 (Fig. 4L). Cytokeratin staining of first trimester decidual samples demonstrated an absence of invading trophoblast within the sections analyzed (data not shown).

Amounts of total furin protein do not alter during the cycle or decidualization

There were no significant differences in the levels of MB&S furin present in endometrial glands between different phases (Fig. 5A). However, significantly less MB furin was detected during the LS phase compared with the proliferative phase ($P < 0.05$; Fig. 5B). The scarcity of luminal epithelium on sections did not allow for consistent scoring. However, wherever there was luminal epithelium present, the staining was equivalent to that of endometrial glands. In stromal fibroblasts, MB&S furin levels were high throughout the cycle and in first trimester decidua (Fig. 5C). Nevertheless, there was less MB furin during the MS phase of the cycle than during menstruation (Fig. 5D; $P < 0.05$). Semi-quantitative assessment of the percentage of cells staining at any intensity returned the same results as H scoring (data not shown).

PC7 and PACE4 proteins are immunolocalized to stromal and glandular compartments throughout the cycle

PACE4 and PC7 antibodies failed to precipitate sufficient amounts of their respective proteins from endometrial protein extracts for detection of these PCs on western blots of endometrial protein extracts. However, both antibodies are affinity purified, and pre-incubation in blocking peptide obliterated PACE4 immunostain. Non-immune IgG controls were also clearly negative.

PACE4 and PC7 immunostains were observed at low to moderate intensities in stromal and glandular compartments (Fig. 6). Serial staining for PACE4 and leukocyte common antigen (Fig. 6G and H) confirmed stromal fibroblasts as the main staining cells within the stroma. Variation in staining intensities in glands or stroma was not apparent across the cycle or with decidualization. Rather, PACE4 and PC7 protein expression seemed to be constitutive.

Discussion

This study investigated the expression of furin, PC1/3, PC2, PC4, PACE4, and PC7/8 in the human endometrium, with particular emphasis on the spatio-temporal expression of furin. Furin, PC4, PACE4, and PC7 are transcribed in normal cycling endometrium. However, expression of PC1 and PC2 is negligible. Furin is transcribed throughout the menstrual cycle and in first trimester decidua with transcript levels being highest during menstruation and lowest during proliferation. Furin and PC7 mRNA levels did not alter with decidualization of ESCs *in vitro*.

Figure 5 H-scores (mean ± S.E.M.) for membrane-bound (MB) and total furin (MB&S) in endometrial glands (A and B) and stromal fibroblasts (C and D). Levels of total furin were high in endometrial glands throughout the cycle and during early pregnancy (A). Levels of MB furin in endometrial glands declined during the late secretory phase (B). Levels of MB&S furin were unaltered during cycle and early pregnancy in stromal fibroblasts (C). Amounts of MB furin declined in stromal fibroblasts towards the mid-secretory phase (D). Well-decidualized stromal cells (striped column) stained strongly for furin. M, menstrual; P, proliferative; ES, early secretory; MS, mid secretory; LS, late secretory phase; D, first trimester pregnancy. * $P < 0.05$.

Figure 6 Histological sections of proliferative (A, B and L), mid-secretory (C, D, G, H, M, N and Q), late secretory phase (E, F, O, P and R) and early pregnant (O and P) endometrium immunostained for PACE4 (free antibody: A, C, E and G, or blocked with immunogen: B, D, F and H), leukocyte common antigen (H, serial to G) and PC7 (free antibody: L, M and O or blocked with immunogen: N and P). Competition blocking with the immunogen significantly diminished staining intensities and sections were clear of any stain following incubation with equivalent concentrations of non-immune goat IgG (Q and R). Scale bars = 50 μ m.

Immunoprecipitation and immunoblotting confirmed that the antibodies used for immunohistochemistry detect active and latent forms of MB furin. We confirmed that antibody sc-20801 does not detect PC4 and PC6 despite similarities of the immunogen, since no bands were observed at 72 and 120 kDa as required for PC4 and PC6 detection. Homologies of the immunogen with other PC family members are negligible. Although specific detection of soluble furin could not be verified, the different temporal staining patterns using antibodies directed against either MB or total furin (MB&S) suggest that the soluble isoform is detected. Antibodies against MB furin, PC7 and PACE4 are raised against peptides of negligible homology with other PC family members. Specificity of PACE4 and PC7 immunostain was confirmed through significant diminution of any signal during competition blocking with the immunogen.

Furin protein was localized in stromal fibroblasts, glandular and luminal epithelium, endometrial vascular

endothelia, and some leukocytes throughout the cycle and during first trimester decidualization, although double staining demonstrated that stromal staining was in fibroblasts. Although both glands and stroma contained MB and MB&S furin at all times, MB furin declined during the LS phase in glands and during the MS phase in stromal fibroblasts. This suggests enhanced cleavage of MB furin to produce soluble furin during the secretory phase, which, however, requires further experimental validation.

PACE4 and PC7 protein were immunolocalized to stromal and glandular compartments throughout the cycle and during first trimester pregnancy, suggesting constitutive expression.

The recent discovery of a PC, PC6, as a key factor required for endometrial preparation for implantation (Nie *et al.* 2005a, Okada *et al.* 2005) raised the question whether other PCs might have similar importance for implantation and endometrial physiology. The data

provided here do not support such roles. In contrast to PC6, furin and PC7 transcription are not up-regulated with decidualization of ESCs *in vitro*. Furthermore, furin, PACE4, and PC7 proteins are constitutively expressed in endometrial glands throughout the cycle and not up-regulated during the secretory phase like PC6. Stromal expression of these proteins is not restricted to decidualized cells like PC6. Similarly, decidualization experiments in the mouse have shown that while PC6 mRNA is up-regulated with decidualization and is essential for implantation, furin, PC7, and PACE4 are independent of decidualization (Tang *et al.* 2005). Whereas furin, PACE4, and PC7 might be important for general cellular regulation in the endometrium, PC6 is apparently specialized for endometrial preparation for implantation. This indicates its potential use as a contraceptive target. However, constitutive expression of other PCs that are structurally highly homologous would need to be taken into consideration in the design of a therapeutically useful inhibitor.

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