Expression of activin receptors, follistatin and betaglycan by human endometrial stromal cells; consistent with a role for activins during decidualization

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Decidualization of the human endometrium is critical for implantation, but the mechanisms involved are largely unknown. Activin subunits are expressed in endometrium during decidualization. From its known actions in cell differentiation and tissue remodelling, we hypothesized that activin A is involved in the paracrine regulation of decidualization. We examined the expression of activin receptors (ActRs) by semi-quantitative and real-time RT–PCR. mRNA for all ActR subtypes (Ia, Ib, IIA and IIB) was detected in endometrium, with maximal expression in the early secretory phase and in early pregnancy. ActR protein was localized exclusively to stromal and endothelial cells. This expression pattern was confirmed by in-situ hybridization. Activin bioavailability is locally regulated by its binding protein, follistatin, and also by the antagonist, inhibin. Inhibin competition for ActRII binding is enhanced by the binding protein, betaglycan. Follistatin and betaglycan were also detected in the endometrium, localized to stromal and epithelial cells. This co-expression of activin subunits, receptors and binding proteins indicates that stromal cells are capable of responding to activin, and that there is tight local regulation of activin action within the endometrium. As activin production is up-regulated in decidual cells, this provides further evidence for an involvement of activins during stromal cell decidualization.

Key words: activin receptors/betaglycan/decidualization/follistatin/implantation

Introduction

The functionalis layer of the human endometrium undergoes dramatic morphological and functional changes during every menstrual cycle (Loke and King, 1995). During the mid to late secretory phase, endometrial stromal cells differentiate to form decidual cells, become enlarged and rounded and deposit a pericellular membrane. These cells are functionally distinct and produce a wide variety of growth factors and cytokines, which are believed to promote decidualization and regulate trophoblast invasion. These changes are initiated in stromal cells immediately underlying spiral arterioles and spread progressively throughout the endometrium if pregnancy is achieved (Bell, 1991). Decidualization is an essential preparative event for blastocyst implantation and establishment of pregnancy. Although it is driven by progesterone (Psychoyos, 1973), stromal cell decidualization is known to be facilitated by a number of endometrially-derived paracrine agents, including prostaglandin E2 (PGE2) (Frank et al., 1994; Lim et al., 1997), interleukin-11 (Robb et al., 1998; Dimitriadis et al., 2001), corticotrophin-releasing hormone (Zoumakis et al., 2000) and other ligands acting through the cAMP pathway (Tang et al., 1994). However, the exact cellular and molecular mechanisms remain largely unknown, and there is a need to fully characterize the paracrine factors involved in decidualization in order to understand the mechanisms of endometrial receptivity.

Activin A is a candidate paracrine agent for promotion of stromal cell decidualization. Activin βA and βB subunits are produced by human endometrium, predominantly by epithelial cells in non-pregnant endometrium (Leung et al., 1998; Petraglia et al., 1998; Jones et al., 2000). Following decidualization in the mid to late secretory phase, stromal cells begin to express mRNA and protein for activin subunits, and this continues into early pregnancy (Otani et al., 1998; Jones et al., 2000). Furthermore, both epithelial cells (Petraglia et al., 1998) and decidualized stromal cells (Jones et al., 2001) have the capacity to secrete high concentrations of dimeric activin A in vitro. Although the functions of endometrial-derived activins are not yet known, many of the known paracrine actions of activin A in regulating cell differentiation, tissue remodelling and inflammatory processes (Mather et al., 1992; Yu and Dolter, 1997; Münz et al., 1999) are consistent with events during decidualization. This has led us to hypothesize that

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endometrially-derived activins are regulators of stromal cell decidualization. To address this question further, it is important to establish whether the endometrium is capable of responding to activins, and to investigate in particular the expression of activin receptors (ActRs) by endometrial cells and the presence of extracellular regulators of activin action, follistatin and inhibin.

Activins elicit a cellular response through interaction with specific cell surface serine/threonine kinase receptors of the transforming growth factor (TGF)-β superfamily (Hoodless and Wrana, 1998; Mummery and Van Den Eijnden-Van Raaij, 1999). Two subtypes, types I and II, of ActRs exist. Activin binds to a type II receptor (either ActRIα or ActRIβ), which recruits and activates a type I receptor [ActRIα (ALK-2; activin receptor-like kinase-2) or ActRIβ (ALK-4)]. Type I receptors initiate a signalling cascade through phosphorylation of intracellular Smads which translocate to the nucleus and promote gene expression (Massague and Chen, 2000).

The activity of activin is tightly regulated by the local extracellular expression of follistatin, a structurally unrelated protein, which binds with high affinity to activin (Shimonaka et al., 1991) and neutralizes its activity (de Winter et al., 1996). The affinity of activin for follistatin is similar to that for its receptors (Schneyer et al., 1994); thus, it plays a major role in regulating activin bioavailability in the circulation and within tissues (Phillips and de Kretser, 1998).

Inhibins act as an activin antagonist, and as decidualized stromal cells co-express both inhibin/activin α and β subunits, they thus have the capacity to produce both inhibin and activin dimers. No specific inhibin signalling receptor has been identified to date (Robertson et al., 2000); however, inhibin A can compete with activin for binding to type II receptors, forming a non-signalling complex (Xu et al., 1995). The recent discovery that betaglycan, the TGFβ type III receptor, binds inhibin with high specificity and enhances its interaction with the activin type II receptor (Lewis et al., 2000), reinforces the theory that inhibin acts as an activin antagonist.

To investigate the potential actions of activins and inhibins in the decidualization of the endometrium, we have examined the expression of ActRIα, 1b, 1la and 1lb, and of the binding proteins follistatin and betaglycan in samples of human endometrium taken across the menstrual cycle and in early pregnancy. These studies provide further information regarding the responsiveness of endometrial cells to activins and inhibins, which in turn gives vital information regarding potential functions of activins in the uterus, particularly during decidualization.

### Materials and methods

**Tissue collection**

Endometrial biopsies (n = 40), representative of all stages of the menstrual cycle, were collected by dilatation and curettage from women undergoing laparoscopy. All women were <40 years of age, experiencing regular menstrual cycles, and had not taken hormonal contraceptives in the past 90 days. The stage of the menstrual cycle was initially determined from the number of days since the last menstrual period and was subsequently confirmed by histological dating. First trimester decidua (n = 7) was collected prior to termination of pregnancy (6–8 weeks amenorrhoea). Term placenta was collected (n = 3) following delivery by Caesarean section. All tissue collections were approved by the appropriate Institutional Human Ethics Committee. Written informed consent was obtained from all women participating in the study.

Tissue samples were fixed immediately in 4% buffered formalin overnight at 4°C, then washed thoroughly with Tris-buffered saline (TBS, pH 7.6) prior to routine paraffin embedding. A further piece of tissue was transported in RNA Later (Ambion, Austin, USA) for RNA extraction.

**RNA extraction and cDNA synthesis**

Total RNA was extracted from endometrial samples from each menstrual cycle phase (n = 3–6 per stage: menstrual, proliferative, early, mid and late secretory) and early pregnancy by acid guanidinium thiocyanate–phenol–chloroform extraction (Sigma Diagnostics, St Louis, USA). Term placenta was used as a positive control. RNA was then treated with ribonuclease (RNase)-free deoxyribonuclease (DNase; Ambion) to remove contaminating genomic DNA. The concentration of RNA in the final preparation was determined spectrophotometrically, and the RNA quality was evaluated by gel electrophoresis (1% agarose) and by the ratio of optical density by calculation of OD_{260–280} (1.8–2.0). Total RNA (2 µg) was reverse transcribed at 46°C for 1.5 h in 20 µl reaction mixture using 100 ng random hexanucleotide primers and 6 IU AMV reverse transcriptase (Roche). Negative controls were performed by omission of reverse transcriptase.

**Semi-quantitative PCR**

Semi-quantitative PCR for mRNA expression of all ActR subtypes and binding proteins was performed using a conventional PCR block cycler (Hybaid, Middlesex, UK). Equal loading was monitored by GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression. A 1.5 µl aliquot of RT product was amplified in a total volume of 50 µl using 1.5 µl of RT single strength PCR buffer (10 mmol/l Tris–HCl, 1.5 mmol/l MgCl₂, 50 mmol/l KCl, pH 8.3; Roche), 2.5 mmol/l dNTPs (Gibco, Melbourne, Australia), 15 pmol sense and antisense primers (Table I), and 2.5 IU Taq DNA polymerase (Roche). The PCR was performed in three stages as follows: the first stage involved

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ActRIα</td>
<td>5'-GCACATAGATATTTGGGACC-3'</td>
</tr>
<tr>
<td>ActRIβ</td>
<td>5'-ACGTAGAGCTTGAGTCTGC-3'</td>
</tr>
<tr>
<td>ActRIα</td>
<td>5'-CCCTTTTTGGTCGCAC-3'</td>
</tr>
<tr>
<td>ActRIβ</td>
<td>5'-ATCCAACGGAGCCCTGCTCAT-3'</td>
</tr>
<tr>
<td>ActRIα</td>
<td>5'-AACCATGTCATAGGATGTCGC-3'</td>
</tr>
<tr>
<td>ActRIβ</td>
<td>5'-CTTTACCTACATCCAGCTGCTG-3'</td>
</tr>
<tr>
<td>ActRIβ</td>
<td>5'-CACGTGGGCACAGCGTAC-3'</td>
</tr>
<tr>
<td>ActRIα</td>
<td>5'-CTGATGTCACCGAGCTGCTG-3'</td>
</tr>
<tr>
<td>Betaglycan</td>
<td>5'-ACATGGATAAGAGCATTACG-3'</td>
</tr>
<tr>
<td>Follistatin</td>
<td>5'-GTTTCTGTCCAGGCACTCAC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CAGAGTTCTTCCTTTAATCTTG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GATGTACCTTAGGACCTTGTGC-3'</td>
</tr>
</tbody>
</table>

*R^Drummond et al. (2001); ^Fuller et al. (2002); ^Nie et al. (1999).
95°C for 3 min, x°C for 3 min, where x is the annealing temperature for the individual primer pairs (Table II), and 72°C for 3 min; the second stage involved 28–33 cycles of 95°C for 1 min, x°C for 1 min, and 72°C for 1 min; and the final stage was 72°C for 10 min. Annealing temperatures and cycle number were optimized for each primer set (Table II) to ensure that amplification was in the exponential phase. PCR products were analysed by electrophoresis on a 1% agarose gel (Roche) and stained with ethidium bromide. Bands of interest were excised from the gel, purified and directly sequenced to confirm their identity.

**Real-time PCR**

To further investigate potential fluctuations in ActR mRNA content at different stages of the menstrual cycle, real-time PCR was performed using a Roche LightCycler. Products were analysed at the log-linear portion of the amplification curve to identify the crossing point for each sample (represented as cycle number of intersection of best fit line through the log-linear region). cDNA generated from a single decidual sample from early pregnancy was selected for use as a standard for all PCR reactions and was serially diluted 1:2, 1:20, 1:100, 1:200 and allocated arbitrary concentrations of 1.0, 0.1, 0.02, 0.01. Sample cDNA was diluted 1:2–1:10 in sterile water for analysis on the LightCycler. Concentrations of mRNA for ActRs and binding proteins were calculated relative to the standard curve, and adjusted for GAPDH expression levels.

The cDNA template (2 µl) was added to sterile capillaries to a total volume of 20 µl, containing PCR mastermix (Roche), including SYBR Green I, dNTPs, Taq enzyme and reaction buffer, supplemented with optimal concentrations of MgCl₂ (Table II) and specific primers (5 pmol). An initial denaturing step was performed for 10 min at 95°C, prior to 40 cycles of 95°C for 15 s, x°C for 5 s and 72°C for 15–24s (elongation time specific to primer pair; Table II). Fluorescence was monitored continuously during cycling at the end of each elongation phase. Fluorescence was acquired at a temperature of 87°C for ActRIB, above the melting temperature of primer dimers, enabling the measurement of specific PCR product alone. At the end of each program, melting curve analysis was carried out to ensure specificity of the reaction products (Table II). The sizes of the products were also confirmed by gel electrophoresis for selected samples.

Whenever possible samples were analysed within the same run to reduce the variability between assays. Samples were initially run in duplicate and the intra-assay variation between samples run in triplicate for each primer pair was <2%.

**Statistical analysis**

To determine if there were significant differences in mRNA expression level for the ActR subtypes and binding proteins at the different stages of the menstrual cycle and early pregnancy, data were subjected to analysis of variance with Tukey’s post hoc test. A value of $P < 0.05$ was considered statistically significant.

**Immunohistochemistry**

Immunohistochemistry was conducted using specific goat polyclonal antibodies raised against human activin receptors and betaglycan (R&D Systems, Minneapolis, USA). Specificity has previously been confirmed by immunohistochemistry and Western blotting against native placental and endometrial proteins (Manuelpillai et al., 2001; Schneider-Kolsky et al., 2001). Paraffin sections (5 µm) of endometrium from all stages of the menstrual cycle ($n = 40$) and early pregnancy ($n = 7$) were dewaxed in histol and rehydrated through descending grades of ethanol. Endogenous peroxidase activity was quenched by immersion in 3% H₂O₂ for 10 min. Sections were then incubated with blocking solution containing 10% horse serum, 2% human serum and 0.1% Tween-20 in TBS for 30 min. Primary antibodies were applied diluted to 0.5 µg/ml (ActRIa), 2 µg/ml (ActRIB), 2.5 µg/ml (ActRIBa) and 0.6 µg/ml (ActRIBb) or 2 µg/ml (betaglycan) in blocking solution, for 1 h at room temperature for ActR or at 37°C for ActRIB, IIB and IIB. Antibody localization was detected by sequential application of biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, USA) in blocking solution, and an avidin–biotin–complex conjugated to horse-radish peroxidase (Dako, Glostrup, Denmark). The substrate used was diaminobenzidine (Zymed, San Francisco, USA), which forms an insoluble brown precipitate, and nuclei were counterstained blue with Harris’ haematoxylin (Sigma). A similar protocol was utilized for detection of follistatin, which was localized using a monoclonal antibody specific to the short form, FS-288 (FS17/2) (Evans et al., 1998). A microwave antigen retrieval step was performed prior to incubation with the primary antibody, and the secondary antibody used was biotinylated horse anti-mouse (Vector). Positive control tissues used were term placenta for activin receptors (Manuelpillai et al., 2001) and follistatin (Petraglia et al., 1994) and rat ovary for betaglycan (Drummond et al., 2001). A negative control was included for each tissue section by substitution of the primary antibody with a matching concentration of goat IgG.

**Northern hybridization**

ActRIa, IIB, IIB and follistatin mRNAs were detected by Northern hybridization using cDNA probes (468, 504, 348 and 300 bp respectively) against bovine activin receptor and follistatin sequences which possess a high degree of homology to the human sequence (Matzuk and Bradley, 1992; Feijen et al., 1994; Verschueren et al., 1995). ActRIB mRNA was detected using a 551 bp cDNA probe against the human ActRIB subtype, synthesized by subcloning of a PCR product generated from HepG2 cDNA into PGE-T plasmid (Promega), and its identity was confirmed by DNA sequencing.

Total RNA (10 µg) from control samples bovine corpus luteum (CL) (Singh and Adams, 1998), HepG2 cells (human hepatoma cell line) and representative endometrial samples were denatured and separated by gel electrophoresis on a 1% agarose gel (containing 0.66 mol/l formaldehyde, 1×3-morpholinopropansulfonic acid

### Table II. Details of PCR amplification conditions for each primer pair for both conventional and real-time PCR

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Product size (bp)</th>
<th>Annealing temp. (°C)</th>
<th>No. of cycles</th>
<th>MgCl₂ conc. real-time PCR (mmol/l)</th>
<th>Extension time real-time PCR (s)</th>
<th>Melting temp. of PCR product (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ActRIa</td>
<td>513</td>
<td>64</td>
<td>28</td>
<td>3</td>
<td>20</td>
<td>87.4</td>
</tr>
<tr>
<td>ActRIB</td>
<td>290</td>
<td>58</td>
<td>30</td>
<td>2</td>
<td>15</td>
<td>87.0</td>
</tr>
<tr>
<td>ActRIla</td>
<td>551</td>
<td>64</td>
<td>28</td>
<td>4</td>
<td>24</td>
<td>85.6</td>
</tr>
<tr>
<td>ActRIBb</td>
<td>320</td>
<td>64</td>
<td>30</td>
<td>2</td>
<td>15</td>
<td>90.4</td>
</tr>
<tr>
<td>Follistatin</td>
<td>500</td>
<td>64</td>
<td>31</td>
<td>3</td>
<td>20</td>
<td>88.3</td>
</tr>
<tr>
<td>Betaglycan</td>
<td>320</td>
<td>64</td>
<td>33</td>
<td>3</td>
<td>15</td>
<td>87.9</td>
</tr>
<tr>
<td>GAPDH</td>
<td>385</td>
<td>60</td>
<td>30</td>
<td>4</td>
<td>15</td>
<td>88.9</td>
</tr>
</tbody>
</table>
treated identically to act as negative controls. Third trimester placenta were applied at matching concentrations to serial tissue sections and sections were mounted with Glycerol Gelatin (Sigma). Sense probes to produce a deep blue nitro blue tetrazolium chloride (NBT/BCIP) (Dako) as a chromogen, 0.1% Triton-X-100), with 5-bromo-4-chloro-3-indoxyl phosphate/-blocking serum (10% normal sheep serum, 10% fetal calf serum, antibody conjugated to alkaline phosphatase (Roche) diluted in
bound probe was subsequently visualized using an anti-digoxygenin treatment with 20 µl NaCl, 10% blocking reagent for nucleic acid hybridization; Roche) at 68°C. The blot was then hybridized for 17 h at 68°C with a cDNA probe labelled with [α32P]digoxygenin-dCTP (Amersham) by random priming. Repeated post-hybridization washes were also conducted at 68°C in SSC buffers of ascending stringency, from 2× to 0.1×SSC, containing 0.1% SDS. For autoradiography, Kodak X-OMAT(AR) film (Kodak, Melbourne, Australia) was used, in conjunction with an intensifying screen, at ~80°C for between 4 h and 1 week. Nylon membranes were stripped of probe by boiling in 0.1% SDS, 1 mmol/l EDTA, and reprobed for GAPDH cDNA probes as previously described (Salamonsen et al., 1997) to allow comparison of mRNA abundance in the same samples (data not shown).

**In-situ hybridization**

In-situ hybridization was conducted using digoxygenin-labelled riboprobes to localize mRNA encoding ActRla, la, Ia and Ib subtypes and follistatin in endometrial samples. Riboprobes were synthesized from the same plasmid preparations used for Northern hybridization, using DIG RNA labelling mix (Roche) with SP6/T7/T3 polymerase, and were thereafter purified by centrifugation through ChromaSpin columns (Clontec, Palo Alto, USA), quantitated and stored at ~80°C until further use.

Paraffin sections of 5 µm were dewaxed in histosol and rehydrated through ascending grades of ethanol. Tissue sections were thereupon digested with 12 µg/ml proteinase K for 30 min at 37°C. Following post-fixation with 4% paraformaldehyde for 10 min at 4°C, tissue sections were washed thoroughly in 0.1 mol/l phosphate-buffered saline, dehydrated through ascending grades of ethanol and air dried. Antisense and sense DIG-labelled probes (1 ng/µl) were applied in hybridization buffer (containing 2×SSC, 10% dextran sulphate, 0.01% sheared salmon sperm DNA (Gibco), 0.02% SDS (Sigma), 50% formamide (Sigma)) and incubated for 17 h at 42°C. Post-hybridization, tissue sections were washed four times in 0.1×SSC at 42°C in a shaking waterbath, and non-specific binding was abolished by treatment with 20 µg/ml RNase A for 30 min at 37°C. Specifically bound probe was subsequently visualized using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche) diluted in blocking serum (10% normal sheep serum, 10% fetal calf serum, 0.1% Triton-X-100), with 5-bromo-4-chloro-3-indoxyl phosphate/ nitro blue tetrazolium chloride (NBT/BCIP) (Dako) as a chromogen, to produce a deep blue–purple stain. No counterstain was used and sections were mounted with Glycerol Gelatin (Sigma). Sense probes were applied at matching concentrations to serial tissue sections and treated identically to act as negative controls. Third trimester placenta and amniochorion tissue sections were included as positive controls.

**Results**

**RT–PCR**

Endometrial and decidual total RNA was analysed for expression of all ActR subtypes, follistatin and betaglycan by RT–PCR, using GAPDH as a housekeeping gene. Following gel electrophoresis, positive control human term placenta showed bands of 513, 290, 551, 320, 500 and 320 bp, corresponding to the regions of the human ActRla, Ib, Ia, Ib, betaglycan and follistatin genes amplified (Figure 1). A band of 385 bp was obtained for GAPDH amplification. Representative PCR products were gel excised, purified and DNA sequenced to confirm their identity. No signal was detected when reverse transcriptase was omitted.

By semi-quantitative RT–PCR, expression of all activin receptor subtypes and binding proteins was detected in both early pregnancy and non-pregnant endometrial samples, with cyclical variations evident. All ActR subtypes appeared to be expressed at elevated levels during the early secretory phase and in early pregnancy (Figure 1). Follistatin expression was low across the menstrual cycle, with faint positive signals obtained only from selected late proliferative and early secretory phase endometrial samples. Decidual samples from early pregnancy were found to express follistatin mRNA. Betaglycan mRNA was strongly expressed in decidual samples from early pregnancy, and was elevated in mid-cycle non-pregnant endometrial samples.

To further investigate these potential variations, mRNA expression was quantitated using real-time PCR. With the standard decidual cDNA, a single band of expected size was
Figure 2. Real-time PCR results. Quantitation of mRNA expression patterns across the menstrual cycle and early pregnancy.

(A) Representative PCR products for each receptor subtype and binding protein amplified from a decidual sample used as a standard for all subsequent reactions. (B) Histograms illustrating fluctuations in mRNA expression (mean values ± SEM) for activin receptors across the menstrual cycle (P = proliferative, n = 5; ES = early secretory, n = 3; MLS = mid to late secretory, n = 5; PREG = early pregnancy, n = 6). Significantly elevated expression of ActRIa and Ib mRNA was detected in the early secretory phase compared with the proliferative or mid/late secretory phase, while ActRIIa mRNA was elevated in the early secretory phase only when compared with the mid/late secretory phase. Maximal expression of both follistatin and betaglycan were detectable in decidua from early pregnancy, with respect to proliferative and mid/late secretory phases, while an additional peak of expression for follistatin mRNA occurred in the early secretory phase. * \( P < 0.05; ++ \ P > 0.01 \).

detectable for each primer pair following gel electrophoresis (Figure 2A). The concentration of each PCR product was calculated by comparison with a standard curve from decidual cDNA in arbitrary units and corrected for expression of internal standard GAPDH. Whilst this method does not give absolute copy numbers of mRNA transcripts, the relative expression levels of an individual receptor subtype can be compared between the different stages of the menstrual cycle. Concentrations of mRNA transcripts for ActRIa, Ib and IIa were significantly elevated \( (P < 0.05) \) in the early secretory phase.
of the menstrual cycle when compared with expression levels during the proliferative and/or mid/late secretory phases (Figure 2B). A high degree of variability was detected in ActRIa and ActR Ib mRNA expression during early pregnancy, reflecting the heterogeneous nature of the tissue in terms of extent of decidual progression (Bell, 1991). Despite this heterogeneity, there was a non-significant trend towards elevated expression of both type I receptors in the early pregnancy samples. ActRIIb concentrations did not vary significantly between any stages of the menstrual cycle or early pregnancy.

To directly compare the relative abundance of the individual type I and II receptor subtypes, the threshold crossing points were analysed at the stages with maximal expression, i.e. early secretory phase and early pregnancy (Figure 3). This is the cycle number at which the signal is detectable above background noise and is inversely related to expression level, with a higher number of mRNA transcripts requiring fewer cycles of amplification. At both stages, ActRIb appeared to be the most abundant type I receptor, although this only reached statistical significance in early pregnancy. Similarly, differential expression of the type II receptors was apparent, with a significantly greater number of amplification cycles required for detection of ActRIIb compared with ActRIIa in early secretory phase endometrium. This indicates that ActRIIa is the more abundant type II receptor.

The pattern observed for follistatin expression by semi-quantitative RT–PCR was confirmed by real-time PCR, with two significant peaks of expression apparent (P < 0.05), again in the early secretory phase and in early pregnancy (Figure 2B). Betaglycan mRNA expression was also up-regulated (P < 0.05) in the decidua of early pregnancy compared with non-pregnant endometrium in the proliferative and mid/late secretory phases (Figure 2B).

**Immunohistochemistry**

Activin receptor protein was detected in endometrium (Figure 4A–F), with localization of all subtypes to stromal cells in both non-pregnant and pregnant tissues. No staining was detected in either surface or glandular epithelium at any stage of the cycle. Immunostaining for ActR protein was found in the early secretory phase and persisted in the mid to late secretory phases, when mRNA was found to be down-regulated, with the exception of endometrium collected immediately prior to and during menstruation. Additional intense staining was observed for all subtypes in endothelial cells of many different blood vessel types, including small capillaries and veins in the subepithelial plexus (Figure 4L,M). Term placental sections included as a positive control exhibited intense immunostaining in endothelial cells of blood vessels (Figure 4N), as expected (Manuelpillai et al., 2001). No staining was detected when primary antibody was replaced with matching concentrations of goat IgG (Figure 4L,N inset).

Immunostaining for the short form of follistatin, FS-288, was observed in amniochorion (Figure 4O) and in the syncytiotrophoblast of term placenta, consistent with published data (Petraglia et al., 1994). An intense punctate pattern of staining was detected for FS-288 in glandular epithelium in non-pregnant and pregnant endometrium (Figure 4G,J). From the distribution along the apical region of the glandular epithelial cells in secretory phase endometrium, these are believed to be secretory vesicles. Additional immunostaining was detected in a subset of decidualized stromal cells (Figure 4K) and vascular endothelial cells (not shown). Low levels of staining for FS-288 were observed in the menstrual and proliferative phases compared with endometrium collected from the early secretory phase and early pregnancy decidua.

Expression of the inhibin binding protein, betaglycan, was also detected in endometrium by immunohistochemistry. Betaglycan was also localized predominantly to stromal cells (Figure 4H), but there was additional staining in a subset of epithelial cells (not shown). Intense immunostaining was observed in decidualized cells during early pregnancy. In placenta, strong immunostaining was identified on the apical surface of the syncytiotrophoblast layer (Figure 4P), and in postnatal rat ovary staining was localized to oocytes and granulosa cells (Drummond et al., 2002; data not shown).

**Northern analysis and in-situ hybridization**

To confirm the suitability and specificity of the bovine probes against activin receptors and follistatin for use for in-situ hybridization on human tissues, Northern analysis was conducted using RNA from HepG2 cells, bovine CL (Figure 5) and selected endometrial samples (data not shown). For ActRIa a signal of 4 kb was detected in bovine CL and HepG2; for ActRIIa, a band of 5 kb was observed in both bovine CL and HepG2, and for ActRIIb multiple bands of 2, 2.5 and 10 kb were detected in HepG2 RNA, but just a single band of 10 kb was seen in bovine CL and decidua/placenta. Two bands of 1.8 and 2.8 kb were detected for follistatin in HepG2 and bovine CL samples. A single band of 4 kb for bovine CL, HepG2 and decidua verified the specificity of the human ActRIb probe. Generally, expression levels of ActRs and follistatin in endometrial samples were below the sensitivity level of the Northern blot technique.

In-situ hybridization was employed to localize mRNA
Figure 4. Immunohistochemical localization of activin receptor subtypes, follistatin and betaglycan in non-pregnant and pregnant endometrium. (A) Immunostaining for activin receptor (ActR)Ia in early secretory phase endometrium and (B) early pregnant decidua, localized to both non-decidualized and decidualized stromal cells. (C) ActRIIb staining in late proliferative phase endometrium. (D) ActRIIa localization in early secretory phase endometrium. (E) ActRIIb immunostaining in mid-secretory phase and (F) early pregnancy. (G) Punctate staining pattern observed for follistatin in a secretory phase sample, demonstrating heterogeneous expression by glandular epithelium. (H) Betaglycan immunostaining in early secretory phase endometrium, localized to stromal cells. (I) Representative negative control for immunostaining by replacement of antisera with goat IgG at a matching concentration. (J) Follistatin ‘vesicles’ at high magnification in an epithelial gland during the secretory phase and (K) diffuse cytoplasmic staining for follistatin in decidual cells during early pregnancy. (L) Endothelial immunostaining for ActRIIa in vessels in the subepithelial plexus in proliferative phase endometrium and (M) ActRIIa in late secretory phase endometrium. Positive control staining for (N) ActRIIa in term placenta, (O) follistatin in amniochorion (P) betaglycan in term placenta. Negative controls are shown in insets. Scale bar on (A) = 50 µm and applies to B, I, J, K, L, M, N and P; scale bar on (C) = 50 µm and applies to D, F, G, H and O; scale bar on (E) = 200 µm.
transcripts for ActRs and follistatin in endometrium using digoxygenin-labelled riboprobes. Expression of all four ActR subtypes was detected, primarily in stromal cells (Figure 5A–E). Very occasionally, staining was detected in glandular epithelium. Follistatin mRNA was detected in a subset of epithelial glands, consistent with the heterogeneous staining patterns observed with immunohistochemistry, and in decidualized stroma (Figure 5F).

Discussion

Investigation into mRNA expression of ActRIa, Ib, Ila and Iib subtypes by human endometrium revealed that levels vary between individual subtypes, and between the different stages of the menstrual cycle and in early pregnancy. Notably, significantly elevated mRNA expression of ActRIa, Ib and Ila subtypes was detected in the early secretory phase of the menstrual cycle, with a trend towards elevated expression in early pregnancy. All ActR subtypes were localized by immunohistochemistry and in-situ hybridization almost exclusively to stromal and endothelial cells. Protein expression of the respective receptors was maintained throughout the secretory phase, with strong staining in decidual cells during the mid to late secretory phase and in early pregnancy. Follistatin and betaglycan were also expressed by human endometrium. Betaglycan mRNA was detectable at greatly elevated levels in pregnant endometrium, whilst follistatin showed peaks of expression in the early secretory phase and early pregnancy, consistent with immunohistochemical findings. These data demonstrate that endometrial stromal cells are capable of responding to activin, and possibly inhibin, and suggest that activin action in the endometrium is under tight local regulation by co-expression of its co-regulators.

To be responsive to activin, a cell must contain both type I and type II receptors. Endometrial stromal cells possess all receptor subtypes, although ActRlb mRNA is expressed at slightly higher levels than ActRIa, whilst expression of ActRIla is significantly elevated compared with ActRIIb. A difference
of one cycle in the crossing point analysis corresponds to a 2-fold change in expression, assuming maximal PCR efficiency, indicating that ActRIb mRNA expression is ~4-fold greater than ActRIa, whilst ActRIIa mRNA expression is ~16- to 36-fold greater than ActRIIb. Whether these changes translate to similar differences in protein concentrations cannot be accurately assessed by immunohistochemistry.

The functional significance of expression of multiple receptor subtypes is not known. Distinct functions for ActRIa and ActRIIb are indicated by different phenotypes in gene deficient mice. Female ActRIIa−/− mice are viable, but fail to reproduce due to a block in folliculogenesis (Matzuk et al., 1995a). Absence of ActRIIb results in neonatal death of most progeny, but those surviving are fertile (Oh and Li, 1997). It is not known whether these differences are related to differential expression or inherent differences in the functions of ActRIa and ActRIIb receptors. In the endometrium, both subtypes have an identical localization, but there is far lower expression of ActRIIb. This is a likely explanation for the lack of uterine phenotype in the ActRIIb null mouse.

Recent evidence suggests ActRIb is the only specific signal-ling receptor for activin. Multiple ligands can interact with ActRIa, including activin, bone morphogenetic proteins (BMPs) and Müllerian inhibitory substance (Clarke et al., 2001; Visser et al., 2001). However, signal transduction in vitro has only been demonstrated via BMP-specific Smads (1, 5, 8) (Macias-Silva et al., 1998). Whilst further investigations are required to verify the specificity of ActRIa in vivo, it seems likely that ActRIb is the critical regulatory factor for activin action in endometrial stromal cells. BMPs 2, 4 and 7 are expressed in mouse decidua (Ying and Zhao, 2000; Paria et al., 2001), indicating functions during early pregnancy. The expression of BMPs in the human uterus has not been examined to date.

Endometrial epithelial cells are the predominant source of activin subunits, but it is likely that epithelial-derived dimeric activin is secreted into the uterine lumen rather than acting on the adjacent stromal layers. Accordingly, the uterine fluid contains high concentrations of activin A (Petraglia et al., 1998). Overall, this suggests that epithelial-derived activin A is involved in functions other than endometrial development. Activin A could be an important endometrial-derived mediator of early placental development, both through promoting trophoblast invasion into decidua (Caniggia et al., 1997) and stimulating placental hormone production (Petraglia et al., 1989; Ni et al., 2000).

Stromal cells also express activin subunits, but only following decidualization (Otani et al., 1998; Jones et al., 2000), a process vital for achieving successful implantation. Stromal cells secrete high concentrations of activin A upon decidualization in vitro (Jones et al., 2001). In this study, we demonstrate the up-regulation of ActR mRNA in stromal cells in the early secretory phase immediately prior to decidualization, and the maintenance of protein production by non-decidualized and decidualized stromal cells throughout the mid-secretory phase and early pregnancy. Therefore, there is the potential for autocrine/paracrine actions of activin A on stromal cells bearing receptors, during and after decidualization.

Activin A has been implicated in the progression of decidualization events, in both rat (Gu et al., 1995; Srivastava et al., 1995) and human uterus (Jones et al., 2000), from the expression patterns of the activin subunits and binding proteins. Many known actions of activin A in actively proliferating and differentiating tissues are consistent with events during stromal decidualization. Activin A may promote endometrial remodeling through modulating the expression of key genes known to be involved in decidualization. For example, activin A stimulates expression and activation of matrix metalloproteinase-2, deposition of constituents of the extracellular matrix, and PGE2 production (Petraglia et al., 1993; Caniggia et al., 1997; Nusing and Barsig, 1999; Keelan et al., 2000). Our demonstration of co-expression of activin and ActRs in decidualizing stromal cells strengthens the hypothesis that activins are important local regulators of stromal decidualization, and indeed we have recent evidence demonstrating that activin A enhances the decidualization of endometrial stromal cells in vitro (Jones et al., 2001).

Activin A is an inhibitor of angiogenesis (McCarthy and Bicknell, 1993), and is constitutively expressed by steady-state endothelial cells. Conversely, follistatin induces angiogenesis, and accordingly is up-regulated during endothelial cell proliferation and migration (Kozian et al., 1997). Angiogenesis occurs in the endometrium both during repair following menstruation and in the formation of the specialized spiral arterioles (Smith, 1998; review). Activin subunits are expressed by endometrial blood vessels (Jones et al., 2000) and here we report that ActRs and follistatin were also localized to endothelial cells. However, no correlation was observed with angiogenic incidence.

FS-288 was expressed by a subset of epithelial glands and decidualized stromal cells, with elevated expression of both mRNA and protein in the early secretory phase and early pregnancy. Whilst full length FS-315 is the most abundant form in the circulation (Schneider et al., 1996), FS-288 exhibits higher neutralizing ability for activin within tissues, when anchored to cell membranes (Inouye et al., 1991). Staining for FS-288 in discrete vesicles, aligned along the apical surface of glandular epithelial cells, implies that these might be secretory vesicles that are released into the uterine lumen. Uterine fluid contains high concentrations of activin A (Petraglia et al., 1998), and thus the epithelially-derived follistatin may be important for restricting the bioavailability of activin within the uterine lumen. Cell surface-anchored FS-288 can act as a clearance factor for activin by internalization and degradation of the FS–activin complex (Hashimoto et al., 1997). Follistatin expression is significantly elevated in early pregnancy, in concert with elevated expression of activin subunits by decidualized stromal cells. The co-localization of follistatin with activin subunits and receptors in decidualized stromal cells is evidence for the tight local regulation of activin action in the peri- and post-implantation phase endometrium. Follistatin may additionally have activin-independent effects, suggested by the distinct phenotypes observed when the follistatin and activin βA subunit genes are deleted or over-expressed (Matzuk et al., 1995a,b; Guo et al., 1998). Follistatin can also bind to other members of the TGFβ superfamily,
including inhibin (Shimonaka et al., 1991) and BMPs 2, 4 and 7 (Yamashita et al., 1995; Fainsod et al., 1997).

The discovery that betaglycan binds inhibin and enhances its interaction with ActR type II (Lewis et al., 2000) was a major breakthrough in understanding how inhibin antagonizes activin action (Gray et al., 2001). Other inhibin-binding proteins have been discovered in inhibin target tissues, p120 (or inhibin-binding protein, InhBP) (Chong et al., 2000) and two potential high affinity inhibin receptors (Farnworth et al., 2001; Harrison et al., 2001). Further characterization of these binding proteins will clarify the mechanisms of inhibin action and uncover any potential signal transduction pathways elicited specifically by inhibin binding (Robertson et al., 2000). We demonstrate here that betaglycan mRNA is expressed by endometrial cells, with maximal expression in early pregnancy. Immunohistochemical localization demonstrated that the protein is predominantly present in stromal cells (the same cells which possess ActR type II), although staining was also observed in a subset of epithelial glands (Jones et al., 2000). Inhibin α subunit is co-expressed with activin β subunits; thus, the endometrium has the capacity to produce dimeric inhibins and activins. The co-expression of betaglycan with ActRs on stromal cells indicates that inhibin may have local actions within the endometrium, and its elevated expression in first trimester decidua indicates that inhibin action might be more important during early pregnancy. The function of inhibin in the endometrium is unknown; however, the strong expression of the inhibin α subunit is co-expressed with activin β subunits in epithelial and stromal cells (Jones et al., 2000); thus the endometrium has the capacity to produce dimeric inhibins and activins, However, activin-independent actions of inhibin within the uterus cannot be ruled out.

Betaglycan also acts as the TGFβ type III binding protein or receptor, and is essential for presenting TGFβs to the type II receptor (Lopez-Casillas et al., 1991). TGFβs are important modulators of endometrial function, particularly in maternal–fetal interactions during implantation and placentation (Godkin and Dore, 1998). As TGFβ type I and II receptors are expressed by endometrial cells and the syncytiotrophoblast of term placenta (Schilling and Yeh, 2000), betaglycan may also be important for enhancing TGFβ signalling and action in the endometrium and placenta.

Our previous research into inhibin and activin production by the endometrium has led to the hypothesis that activins are important mediators of endometrial growth and differentiation (Jones et al., 2000). Here we describe the expression of ActRs by human endometrium and their localization to stromal and endothelial cells. These findings provide further evidence for activin action within the endometrium, and are the vital groundwork for further studies to explore and identify the functions of activins in this tissue. The localization of all receptor subtypes to stromal cells is consistent with a role for activin in regulating stromal cell function, and the elevated expression of ActR mRNAs immediately prior to decidualization, and the persistence of protein production during decidualization, strengthen the hypothesis that activin is involved in the differentiation process. The co-expression of betaglycan and ActRs in endometrial cells is evidence for inhibin action in decidua, and raises the possibility that other members of the TGFβ superfamily may have endometrial functions. Decidualization of the endometrium is critical for regulating trophoblast invasion during implantation and for enabling successful placentational development. Endometrial dysfunction and insufficient placentation are responsible for the high rates of early spontaneous abortion and low implantation rates during IVF. Furthermore, there is strong evidence linking impaired fetal health, caused by inadequate placentational function, with an increased risk of many adult onset diseases (Barker and Clarke, 1997). Determining which factors are important for decidualization is therefore critical for understanding implantation; this information can then be applied to the development of novel contraceptives to block implantation, or to the improvement of existing infertility treatments.

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