Lefty is expressed in mouse endometrium in estrous cycle and peri-implantation period

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BACKGROUND: Reproductive tissues are unique structures that exhibit cyclic stromal remodelling during menstrual cycles in humans. Ebafl/lefty participates in tissue remodelling of human endometrium by induction of matrix metalloproteases (MMPs). METHODS: We describe the temporal expression and spatial distribution of lefty and tissue remodelling events in mouse endometrium. RT-PCR and real-time PCR were used to identify mRNA expression and western blots to analyse Lefty protein. Immunolocalization was performed with specific antibodies and horseradish peroxidase staining. RESULTS: Lefty was expressed in endometrium throughout the estrous cycle. Expression of MMP (MMP-2, -3, -7 and -14) was higher at estrus, metestrus and/or diestrus while collagen content of endometrium decreased in these phases. During pregnancy, lefty levels were higher on days 3–5 and were minimal by day 9. Similarly, expression of endometrial MMP was higher on days 3 and 5 of pregnancy and was low on day 9. During pregnancy, loss of collagen was initiated on day 3, persisted to day 5, and led to a significantly reduced collagen on day 9. Immunoreactive lefty decorated basal laminae, and was associated with extracellular matrix in stroma. CONCLUSIONS: Regulated expression and spatial distribution of lefty in mouse endometrium confines its biological impact on tissues that undergo remodelling during estrous cycle and pregnancy.

Key words: Ebafl/endometrium/estrous cycle/lefty/mouse

Introduction

In humans, some of the most intense tissue remodelling events occur in reproductive organs. Endometrium undergoes sequential morphological and biochemical changes during each cycle in anticipation of embryo implantation. In human endometrium, this includes a period of proliferation (proliferative phase) followed by changes that collectively characterize a progesterone-dominant secretory phase. These changes are initiated immediately after ovulation, and, several days later, prime endometrium for implantation. Failure of endometrium to initiate or progress through the critical events of implantation during this 'window of receptivity' results in pregnancy failure. At the end of the menstrual cycle, when the serum concentration of progesterone falls, the interstitial fibrillar meshwork of human endometrium undergoes focal breakdown and is extensively lysed at menstruation. Basement membranes around glands and blood vessels also undergo fragmentation and disruption. Breakdown of these structures leads to shrinkage of endometrium, and piecemeal loss of functionalis layer. This is followed by tissue shedding and bleeding (Woessner, 1982). Remodelling of reproductive organs also occurs at the time of implantation. The remodelling of endometrium during pregnancy is comprised of a localized dissolution of endometrial extracellular matrix (ECM) around the implanting blastocyst (Edwards et al., 1996; Salamonsen et al., 2002; Herrler et al., 2003). ECM proteins are degraded by a variety of enzymes, but the matrix metalloproteinases (MMPs) are believed to be the primary enzymes involved in ECM remodelling during menstrual tissue shedding and implantation. MMPs are secreted, zinc-containing enzymes that degrade ECM components under physiological conditions and are loosely grouped by their substrate specificity (Woessner, 1982; Alexander and Werb, 1991; Birkedal-Hansen et al., 1993; Hulboy et al., 1997). Secreted as proenzymes, MMPs undergo conversion to a catalytically active form. Once activated, these enzymes degrade collagen, fibronectin, gelatin, elastin as well as the constituents of basement membrane including laminin.

A prevailing hypothesis is that steroid hormones control the homeostasis of endometrial ECM, but the molecular basis of this control has not been thoroughly worked out (Brunet et al., 1999). This remains a critical gap in our knowledge. We identified a protein that appears to be involved in tissue
remodelling required for embryo implantation and menstrual shedding. The expression of this endometrial bleeding-associated factor (ebal/lefty) in humans was minimal during the proliferative, early and mid-secretory phases, but was increased during peri-menstrual and menstrual phases (Kothapalli et al., 1997). Lefty is a member of the transforming growth factor (TGF)-β family of molecules (Kothapalli et al., 1997). Because lefty does not form dimers, it acts as an inhibitor of other members of the TGF-β family such as nodal and activin. Expression of lefty was increased up to ~100-fold during menstruation (Cornet et al., 2002). These findings suggest that lefty might be involved in ECM remodelling, a requisite for menstrual tissue shedding. We recently showed that lefty induced tissue remodelling by induction of collagenolysis by MMPs (Mason et al., 2002). Lefty induced MMP-3 and MMP-7 in human endometrial explants maintained ex vivo (Cornet et al., 2002). Lefty also induced collagenolytic activity in vivo by cells forced to express lefty (Ulloa et al., 2001; Mason et al., 2002). We were interested in extending these observations to a model system, more amenable to experimental manipulation in vivo. Therefore, the current study was carried out to precisely define the temporal expression and spatial distribution profile of lefty and to characterize the tissue remodelling events in mouse endometrium during the estrous cycle and implantation. The results showed that lefty was expressed in mouse endometrium during estrous cycle and pregnancy in a unique spatial distribution pattern consistent with its role in tissue remodelling in endometrium.

**Materials and methods**

**Materials**

All chemicals were from Sigma–Aldrich company (USA) or Fisher Scientific (USA). Reproductive age outbred female CF-1 and CD1 mice were obtained from Charles River Laboratory (USA). Pooled Poly A RNA from human uterus and colon was purchased from BD Biosciences–Clontech (USA). RNAlater™ RNA Stabilization Reagent and RNaseasy Mini Kit were purchased from Qiagen Inc. (USA). Millenium RNA Marker™ Probe Template cDNA and Poly(A) Purist™ purification and MAXScript™ in vitro transcription kits, and NorthernMax™ Formaldehyde-based system for Northern blotting were from Ambion Inc. (USA). pSTblue-1 cloning vector was from Novagen (USA). Avidin–biotin–peroxidase (ABC) kit was from Vector Laboratories (USA). Goat polyclonal antibody to lefty peptide (M-20) mapped to the carboxy terminus of mouse lefty and its blocking peptide were from Santa Cruz Biotechnology, Inc. (USA). The antibody is reactive on western blot analysis and immunohistochemical staining with both mouse and human lefty. The antibody to actin was from Abcam Inc. (USA). Recombinant Human ebal/Lefty-A was obtained from R&D Systems (USA). This recombinant protein was obtained from expression of a DNA sequence encoding the mature human Lefty-A in NS0, a mouse myeloma cell line. Due to glycosylation and proteolytic processing, this recombinant preparation contains three peptides that migrate as 44, 38 and 34 kDa bands in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. The short form of lefty protein, made in E. coli, was also used as a control in the western blot analysis.

**Approvals**

All experiments in animals were conducted in accord with accepted standards of humane animal care and in accord with NIH guidelines. Approval of institutional IACUC was obtained for carrying out the animal procedures used in this study.

**Sample preparation**

Lefty, MMP and collagen were analysed in the endometria of virgin outbred CF-1 mice throughout the estrous cycle and during the first 9 days of pregnancy. The day of the estrous cycle was determined by vaginal smears as described by Snell et al. (1966) and Rugh (1968). The day when the vaginal plug was detected was designated as day 1 of pregnancy. The expression of lefty and MMP was assessed in endometrial tissues obtained from six mice, for each day of estrous cycle and on days 1, 3, 5 and 9 of pregnancy. Each experiment was repeated at least twice to confirm reproducibility of data.

**Northern blot analysis**

For Northern blot analysis, total RNA and poly-A RNA were extracted and pooled from 10 uterine horns of virgin CD1 mice. Mouse uterine horns and segments of colon were immediately placed in RNAlater™ RNA Stabilization Reagent and stored at ~80°C. Total RNA was isolated from tissues using the RNaseasy Mini Kit. PolyA RNA was isolated on the oligo(dT) cellulose from total RNA using the Poly(A) Purist™ mRNA purification Kit following the manufacturer’s protocol. The 32P-labelled cRNA lefty and RNA marker probes were made by in vitro transcription reaction using MAXScript™ in vitro transcription kit. The template used for generation of cRNA lefty probe was the pSTblue-mouse lefty 1 linearized by Sph I. The template was made by inserting coding region of mouse lefty 1 into the EcorV site of the pSTblue-1 cloning vector. The template construct was subjected to DNA sequencing to ensure insertion and orientation. Millenium RNA Marker™ Probe Template cDNA was used for generation of probe for RNA marker. The cRNA probes were precipitated by NH₄Ac/ethanol, rinsed once in 70% ethanol and resuspended in 50 µl of nuclease-free water. Northern blot analysis was performed using the NorthernMax™ Formaldehyde-based system following the manufacturer’s instructions.

**RT–PCR and real-time RT–PCR**

For RT–PCR and real-time PCR, uterine horns were opened longitudinally and the endometrium was scraped from myometrium using a sterile scalpel. On day 9 of pregnancy, the embryo was removed and the decidual underlying the embryo was used for RNA isolation. Tissues were suspended in phosphate-buffered saline (PBS) and pelleted by centrifugation. The pellets were frozen on dry ice and stored at ~80°C. Scraped endometrial tissues were homogenized in a guanidinium thiocyanate–acid phenol solution, and total RNA was extracted as described by Chomczynski and Sacchi (1987). RNA were treated with DNase to remove any potential contaminating DNA. RT–PCR for lefty was performed using primer sets shown in Table I. This was performed on 5 µg of RNA which was reverse-transcribed with Superscript II (In Vitrogen) in 20 µl volume. The cDNA was diluted to 300 µl with TE. Aliquots (1 µl) of the RT products were subjected to PCR in a total volume of 20 µl PCR mix, containing 22 mmol/l Tris–HCl pH 8.4, 55 mmol/l KCl, 1.65 mmol/l MgCl₂, 220 µmol/l dNTP, 22 IU/ml Taq DNA polymerase, with 0.5 µmol/l of adequate paired primers (Table I). A control PCR with actin template DNA was performed in each experiment. After an initial denaturation at 95°C for 5 min, the DNA was amplified through 30 cycles of 30 s at 95°C, 30 s at the optimal annealing temperature of the primer pair and 30 s at 72°C. The reaction was
terminated at 72°C for 10 min. PCR products were stored at 4°C until use. Real-time PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen, USA) and DNA Engine Opticon system (MJ Research, USA).

**SDS–PAGE and western blotting**

The proteins in conditioned media or cell lysates (12–15 μg protein/lane) were fractionated in a 10% denaturing gel together with pre-stained protein ladder (Life Technologies, Inc., USA) and were subsequently blotted onto nitrocellulose membrane in a Mini-Trans-Blot apparatus (Bio-Rad Laboratories, USA). Blots were incubated with goat polyclonal antibody to lefty peptide (M-20) (1–2 μg/ml). The secondary antibody used was anti-mouse IgG–horseradish peroxidase (HRP; Santa Cruz Biotechnology, USA). Bands were detected by chemiluminescence as described by the manufacturer.

**Immunohistochemical staining**

For immunohistochemical staining, uterine horns were placed in OCT medium and frozen in liquid nitrogen. Lefty was localized by immunostaining according to the ABC procedure as described previously (Hsu et al., 1981; Tabibzadeh and Gerber, 1985; Tabibzadeh et al., 1987). Briefly, sections or cells were fixed in 10% buffered formalin (v/v) for 5 min and then washed in PBS. These were incubated with primary and then with secondary antibody at a concentration of 1–2 μg/ml for 30 min at room temperature. This was followed by incubation in ABC complex. Each incubation was carried out at 37°C and was followed by two washes in 0.1 ml/l PBS (pH 7.4). Immunoreactive cells were visualized by incubation of sections in a mixture of 3,3′-diaminobenzidine tetrahydrochloride (DAB)–H₂O₂ which formed a brown precipitate in stained cells.

**Neutralization of lefty antibody by peptide**

In immunohistochemical and western blot analyses, the goat polyclonal antibody to lefty peptide (M-20) was neutralized using the peptide used for immunization of the animals. The neutralization was done according to the manufacturer’s recommendation. Briefly, the antibody was incubated with 5-fold excess of the peptide (20 ng/ml) in 500 μl of PBS overnight at 4°C. The mixture was then used without dilution.

**Histochemical staining and morphometric analysis**

To quantify the amount of collagen in sections of endometria were stained with Mason-Trichrome, which in view of its affinity, casts a blue colour onto collagen fibres. Collagen fibres appeared as cord- or tape-shaped wavy fibres. The abundance of collagen fibres in stained sections was assessed by morphometric analysis as described previously (Mason et al., 2002). The mean total area/pixel density of the trichrome-positive areas was measured per microscopic field, at ×4 magnification. Three separate, randomly selected fields for each sample were examined.

**Results**

**Expression of lefty mRNA in mouse endometrium during estrous cycle and pregnancy**

To determine whether lefty is expressed in mouse uterine horn, we first carried out RT–PCR. Using the set of primers shown in Table I, lefty was detected by RT–PCR in mouse endometrium during estrous cycle and pregnancy. The size of the product amplified by PCR matched that expected for lefty (Table I). The amplified PCR product was sequenced and the sequence of the product matched that of mouse lefty-1 (data not shown). To further substantiate presence of lefty in mouse uterine horns, total RNA from mouse uterine horns (20 μg) and colon (20 μg) was prepared. These were subjected along with poly A RNA from human uterus (2 μg) and colon (2 μg) to hybridization with a 32P-labelled cRNA probe transcribed from a mouse lefty-1 cDNA. A strong positive signal was detected in human uterus and much weaker signals were detected in mouse uterus and colon (data not shown). Poly A RNA from 10 mouse uterine horns and colon were pooled and subjected to Northern blot analysis using the same cRNA probe. Bands of 2.5 and 1.5 kb size were detected in the mouse uterine horn and colon in human uterus. The size of the band detected in human colon was slightly less than 2.5 kb (Figure 1A). When the same amount of poly A was loaded (2 μg), the intensity of the signal detected in the mouse uterine horns was markedly lower than that detected in human uterus. To assess lefty expression during estrous cycle and pregnancy, real-time PCR was performed on total RNA extracted from mouse uterine horns. Lefty was expressed throughout the estrous cycle, with lower levels detected at estrus and on days 1 and 9 of pregnancy (detection of vaginal plug) (Figure 1B and C). Lefty expression was higher on days 3–5 of pregnancy (Figure 1C). There was minimal variation in lefty expression between different samples. However, lefty expression was greatly variable during proestrus (Figure 1B).

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**Table I. Primer sequences used in RT–PCR and real-time PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Corresponding position</th>
<th>Product size (base pair)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lefty-1</td>
<td>Forward: 5′-GCTCCACACACCAGTGGCTTCAC-3′</td>
<td>729–1095</td>
<td>367</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GGCTACCCACAGCAGCAGC-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>Forward: 5′-CAACCCACTAAGGTAGACGC-3′</td>
<td>1176–1539</td>
<td>364</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Forward: 5′-GCAGGTTGAACATCGGAG-3′</td>
<td>514–782</td>
<td>269</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Forward: 5′-TGATGATGAGGACGAGG-3′</td>
<td>646–957</td>
<td>312</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Forward: 5′-GTCCACATCACTAATGCCC-3′</td>
<td>806–1199</td>
<td>394</td>
</tr>
<tr>
<td>MMP-14</td>
<td>Forward: 5′-GATCCACCTTGAAGACACAC-3′</td>
<td>806–1190</td>
<td>385</td>
</tr>
</tbody>
</table>
Lefty protein in mouse endometrium during estrous cycle and pregnancy

To ascertain that lefty is translated in the mouse endometrial tissues, we carried out western blotting on proteins extracted from mouse uterine horns. We recently reported on the processing of lefty proteins (Mason et al., 2002). Culture media of GP⁺E86 cells that were transduced to retrovirally express lefty (LEIG) served as positive control and culture media of GP⁺E86 cells transduced with a control vector (LG) that did not express lefty were used as negative control. Lefty was released from the LEIG cells as a 42 kDa precursor protein and two products of 28 and 34 kDa proteins cleaved from its carboxy terminus. LG cells did not release lefty into culture medium. Lefty was identified by its reactivity with M-20, a commercially available polyclonal goat antibody raised against a synthetic lefty peptide. This antibody detected all three forms of lefty on western blotting including lefty secreted into the culture supernatants of LEIG cells (Figure 2). An additional control consisted of commercially available lefty expressed in a mouse myeloma cell line, NS0. Using the antibody M20 for detection, the prominent band of this lefty appeared as a 38 kDa protein (hLefty, Figure 2). Antibody also detected another control, a 26 kDa short form of lefty made in E. coli (eLefty, Figure 2). M20 reacted with a band slightly larger than the band detected in the culture supernatants of LEIG cells in the proteins extracted from mouse endometrium. These bands were not detected when M20 antibody was omitted or substituted with normal goat IgG, or M20 antibody neutralized with the peptide (Figure 2).

Following these initial series of studies, the proteins extracted from mouse endometria during estrous cycle and pregnancy were subjected to western blotting. Lefty precursor protein was detected in mouse endometrium throughout the estrus cycle, whereas the processed forms of lefty were detected at much lower level during proestrus, estrus and metestrus (Figure 3A). During pregnancy, lefty precursor was

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**Figure 1.** Expression of lefty mRNA in mouse endometrium. Expression of lefty mRNA was analysed in mouse endometrium by northern blot analysis (A) and by real-time PCR during estrous cycle (B) and pregnancy (C). Poly A RNA was prepared from uterine horns and colon of CD1 mice and were subjected along with poly A from human uterus and colon to northern blot analysis. The blot was probed with a 32P-labelled cRNA prepared on a cDNA template of lefty-1. Size of bands was determined by probing the same blot with cRNA probe to RNA markers run in the same gel. Means and SD are shown in B and C.

**Figure 2.** Control studies for western blotting of lefty. Lefty⁻ (LG) and lefty⁺ (LEIG) cells were grown in serum-free medium overnight. Conditioned media of these cells were concentrated in Centricons with 10000 Da threshold. The concentrated media were mixed with SDS lysis buffer and were subjected to western blotting along with recombinant lefty made in myeloma cell line (hLefty), short form of lefty made in E coli (eLefty) and protein extracts derived from a mouse endometrium during estrus (Estrus). Blots were stained using antibody to lefty (M-20), normal goat serum, M-20 antibody to lefty absorbed with blocking peptide before applying it to the blot or with secondary antibody alone.
detected on days 1 and 3, and only a processed form of lefty was detected on day 5. On day 9, lefty precursor or processed proteins were not detectable in endometrium (Figure 3).

**Distribution of immunoreactive lefty in mouse endometrium**

To localize immunoreactive cells in the endometrial tissue, sections of mouse uterine horns were subjected to immunohistochemical staining during estrous cycle and on days 1, 3, 5 and 9 of pregnancy. These cells have been previously characterized (Mason et al., 2002). As reported previously, with anti-lefty antibody, LG cells lacked immunoreactivity for lefty, whereas cytoplasmic immunoreactive protein was found in ~50% LEIG cells (Figure 4A and B). Sections of mouse endometria incubated without lefty antibody, with lefty antibody absorbed with the immunogenic peptide or with IgG did not show staining or showed a very light staining in the glandular and surface epithelium (Figure 4C–H). However, when stained with lefty antibody, staining was detected in several different endometrial compartments (Figure 4I and J). Basal lamina and luminal secretions of endometrial glands exhibited positive immunoreactivity for lefty. In stroma, immunoreactive lefty was found along fibrils deposited among stromal cells and myometrial fibres (Figure 4I and J). Lefty was also present in vessel walls and absent in myometrium (Figure 4J). The staining intensity and distribution pattern did not show significant changes during estrus cycle or on days 1–5 of pregnancy (Figure 5A–G). However, on day 9 of pregnancy, immunoreactivity for lefty was not detectable in endometrium (Figure 3).
Expression of MMP in mouse endometrium during estrous cycle and pregnancy

Using primers shown in Table I, we first confirmed expression of MMP-2, -3, -7, -12 and -14 by RT–PCR, in mouse endometria during estrous cycle and pregnancy. RNA from LEIG cells was used as control in these experiments. Amplicons of expected sizes for MMP-2, -3, -7, -12 and -14 were detected in these samples (Table I, data not shown). All amplified products were sequenced. The sequence of these products matched those of respective MMP. To determine the relative level of expression of MMP, we then carried out real-time PCR (Figure 6). Expression of MMP-2, -3 and -14 peaked at diestrus (Figure 6A). In contrast, the expression of MMP-7 peaked at metestrus. Expression of MMP-12 did not change significantly during estrus cycle (Figure 6A). During pregnancy the expression level of MMP was highest around the time of implantation on day 3 (MMP-12, -14), day 5 (MMP-7) or on both days (MMP-2, -3) (Figure 6B). The expression of all MMP fell on day 9 (Figure 6B).

Collagen content of mouse endometrium in the estrous cycle and pregnancy

Changes in the expression of collagenases are likely to be associated with a corresponding decrease in endometrial collagen. To determine whether changes in expression of MMP were associated with changes in amount of endometrial collagen, the collagen content of endometrial tissue was confined to some vessels in myometrium and little immunoreactivity was present in glands or stroma (Figure 5H).

**Figure 5.** Immunohistochemical staining of lefty in mouse endometrium. Sections of uterine horns of proestrus (A), estrus (B), metestrus (C), diestrus (D), and day 1 (E), 3 (F), 5 (G) and 9 (H) of pregnant uterine horns were stained with M-20 antibody to lefty. L = lumen; G = gland; S = stroma; M = myometrium; L = lumen; V = vessel. (A, I, J) Bar = 50 μm; (B–I) bar = 25 μm.
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Figure 6. Expression of MMP in mouse endometrium. The expression of MMP-2, -3, -7, -12 and -14 mRNA was analysed by real-time PCR in mouse endometrium during estrous cycle (A) and pregnancy (B). Means and SD are shown. P = proestrus; E = estrus; M = metestrus; D = diestrus; 1, 3, 5 and 9 (days of pregnancy).

analyzed. Representative staining of collagen fibres during estrus cycle and quantitative data from analysis of stained sections are presented in Figure 7. Quantitative assessment of the stained fibres showed minimal loss of collagen fibres during the cycle with lower amounts detected at metestrus and diestrus phases (Figure 7A and C). During pregnancy, the amount of collagen fibres was highest at the beginning of pregnancy on day 1 prior to implantation of embryo. Therefore, the amount of endometrial collagen progressively diminished on days 3 and 5, so that only a minimal number of fibres was detected by day 9 (Figure 7B, D).

Discussion

Endometrial tissue breakdown is a unique feature of human endometrium. The menstrual tissue breakdown is associated with the dissolution of the ECM, light-microscopically classified into collagen, reticular and elastic fibres. Significant increase in expression of MMP-3 and MMP-7 is thought to be the leading cause of ECM lysis in human endometrium during menstruation (Goffin et al., 2003). Mouse endometrial tissues, on the other hand, do not undergo the extreme ECM remodelling that leads to menstrual tissue breakdown and bleeding in human endometrium.

We identified a protein, lefty/ebaf, which caused collagenolysis by activation of MMP in human endometrial explants, suggesting that it might be the mediator of endometrial ECM lysis around the time of menstruation (Tabibzadeh, 2002). In this report, we showed that lefty was expressed and that lefty proteins were present in mouse uterine horns during estrous cycle. Lefty proteins were found in the ECM, in the basal lamina around glands, in the interstitium and around vessels. Thus, lefty is strategically positioned and intimately associated with structures in endometrium that are most prone to lysis. Despite its presence, lefty was expressed in mouse uterine horns at a significantly lower level as compared to that expressed in human uterus (Kothapalli et al., 1997). Also, the magnitude of changes which occurred in lefty expression in mouse uterine horn during estrus cycle was much lower than that which occurred in human endometrium during menstrual cycle (Kothapalli et al., 1997; Cornet et al., 2002). We showed that lefty was increased up to 100-fold in human endometrium around the time menstruation whereas expression of lefty was only increased <5-fold in mouse uterine horn from estrus to metestrus, a catabolic stage of the estrous cycle characterized by degenerative changes in the genital tract (Kothapalli et al., 1997; Cornet et al., 2002). In human endometrium, the increase in lefty expression occurred at the same time and at a magnitude similar to that of MMP (Goffin et al., 2003). The level of expression of MMP-3, -7 and MMP-12 increased >100-fold in human endometrium during menstruation (Goffin et al., 2003). In marked contrast, in mouse uterine horns, the magnitude of increase in MMP expression was <6-fold during estrous cycle. The peak of expression of all MMPs, with the exception of MMP-12, was at metestrus (MMP-7) or at diestrus (MMP-2, -3 and -14).

Coordinated with changes in lefty and MMP expression, the collagen fibres were slightly reduced in mouse uterine horns at metestrus and diestrus. Similar to these findings, it was reported that, during metestrus in cycling rats, the wet weight and the collagen content of endometrium decreased by 20% of their proestrus values (Yochim and Blahna, 1976). These findings indicate that, although ECM lysis is an essential feature of mouse endometrium in an undisturbed estrous cycle, the extent of this breakdown is sufficient only to cause regression and not tissue shedding (Marbaix et al., 1996).

Given that mouse endometrium expresses the molecular repertoire required for tissue breakdown, the low level of ECM lysis in mouse endometrium might be related to the low level of lefty and MMP. Significantly higher levels of lefty and MMP might be required to cause tissue shedding in mouse endometrium similar to that which occurs in human endometrium during menstruation. Another factor might involve processing of lefty proteins. Most of lefty protein found in mouse uterine horn during estrous cycle was in a precursor form, suggesting that the precursor might not be processed or that its processed forms were rapidly secreted. Mouse endometrium, however, can be forced to show changes similar to those observed in human endometrium during menstruation and to exhibit tissue shedding. Recently, Brasted et al. (2003) showed that steroid hormone treatment followed by application of a decidualogenic stimulus made mouse endometrium ready to show changes that closely mimicked those which occur in human endometrium at
menstruation. Following these treatments, progesterone withdrawal led to infiltration of endometrium by leukocytes, apoptosis and ultimately led to shedding of decidual zone.

Lefty and MMP were also coordinately expressed during pregnancy in mouse endometrium. Lefty was maximally expressed in mouse endometrium around the time of implantation and its expression subsided on day 9. Similar to these findings, lefty proteins were detectable at the time of implantation and disappeared by day 9 of pregnancy. Interestingly, on day 5 of pregnancy, most if not all of lefty protein was a processed form. MMP including MMP-3 and -7 were also expressed in mouse endometrium during pregnancy (Curry and Osteen, 2003). Expression of MMPs in mouse endometrium increased on days 3 and/or 5 of pregnancy and decreased by day 9. Consistent with these results, Das et al. showed that in mouse uterus, MMP-2 mRNA was highly expressed in the subepithelial stroma on days 3–5 and was primarily expressed in the secondary decidual zone on day 6. MMP-3 mRNA was first detected on day 5 in stromal cells located exclusively at the site of implantation (Das et al., 1997). MMPs are critical to embryo implantation. Injection of peptide hydroxamate MMP inhibitor retarded decidual development. Similarly, development of decidua was inhibited in transgenic mice overexpressing TIMP-1 which inhibits MMP activity (Alexander et al., 1996). MMPs appear to be required for remodelling of collagen network that takes place in response to decidualization and implantation. The process of decidualization is associated with a vanishing collagen types I, III, V and VI content in pregnant uteri (Mulholland et al., 1992; Hurst et al., 1994). Here, we confirmed these findings and showed that during pregnancy, collagen fibres were progressively lost in mouse endometrium from day 3, with relatively less fibres detected by day 9. Several studies showed that this loss of collagen occurred more around the implanting blastocyst (Alexander et al., 1996; Das et al., 1997; Curry and Osteen, 2003). Collagen type I was virtually absent around rat embryos on day 5 of pregnancy, and on days 6–8 of pregnancy very little collagen was left at the primary and secondary decidualized zones (Clark et al., 1992, 1993). Similarly, in humans, collagen types I, III, IV and VI were all reduced during peri-implantation period and were no longer detected in first trimester decidua (Mylona et al., 1995; Iwahashi et al., 1996). Decreased collagen expression has also been described in human decidual tissues in spontaneous abortions (Iwahashi et al., 1996).

In summary, lefty and MMPs are expressed in mouse uterine horns in a coordinated fashion. During estrous cycle, lefty is lower at estrus and higher at metestrus and diestrus. MMP (MMP-2, -3, -7 and -14) are also higher during metestrus and/or diestrus. During estrous cycle, the change in
expression level of lefty and MMPs in mouse endometrium does not occur to the extent sufficient for tissue shedding. During pregnancy, expression of lefty and MMP (MMP-3, -7, -12 and -14) is jointly increased around the time of implantation while their expression is coordinately decreased following implantation on day 9 of pregnancy. Spatially, mouse endometrial lefty is found associated with structures that are prone to tissue remodelling, namely the ECM in stroma and vessel walls and basal lamina around glands. Unique spatial pattern of distribution of lefty in endometrium might be important to its biological effect on ECM, and significant when tissue undergoes remodelling.

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