Secreted phosphoprotein 1 (osteopontin) is expressed by stromal macrophages in cyclic and pregnant endometrium of mice, but is induced by estrogen in luminal epithelium during conceptus attachment for implantation

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Abstract

Secreted phosphoprotein 1 (SPP1, osteopontin) is the most highly upregulated extracellular matrix/adhesion molecule/cytokine in the receptive phase human uterus, and Spp1 null mice manifest decreased pregnancy rates during mid-gestation as compared with wild-type counterparts. We hypothesize that Spp1 is required for proliferation, migration, survival, adhesion, and remodeling of cells at the conceptus–maternal interface. Our objective was to define the temporal/spatial distribution and steroid regulation of Spp1 in mouse uterus during estrous cycle and early gestation. In situ hybridization localized Spp1 to luminal epithelium (LE) and immune cells. LE expression was prominent at proestrus, decreased by estrus, and was nearly undetectable at diestrus. During pregnancy, Spp1 mRNA was not detected in LE until day 4.5 (day 1 = vaginal plug). Spp1-expressing immune cells were scattered within the endometrial stroma throughout the estrous cycle and early pregnancy. Immunoactive Spp1 was prominent at the apical LE surface by day 4.5 of pregnancy and Spp1 protein was also co-localized with subsets of CD45-positive (leukocytes) and F4/80-positive (macrophages) cells. In ovariectomized mice, estrogen, but not progesterone, induced Spp1 mRNA, whereas estrogen plus progesterone did not induce Spp1 in LE. These results establish that estrogen regulates Spp1 in mouse LE and are the first to identify macrophages that produce Spp1 within the peri-implantation endometrium of any species. We suggest that Spp1 at the apical surface of LE provides a mechanism to bridge conceptus to LE during implantation, and that Spp1-positive macrophages within the stroma may be involved in uterine remodeling for conceptus invasion.


Introduction

Implantation involves pregnancy-specific remodeling of extracellular matrix (ECM) and adhesion molecules at the conceptus–maternal interface (Carson et al. 2000, Dey et al. 2004). Adhesion proteins and/or ECM molecules, cell-surface receptors, and growth factors comprise over 20% of the genes that change expression as the human endometrium transforms to an implantation-receptive state (Carson et al. 2002). These molecules are hypothesized to have direct roles in conceptus attachment, invasion, and placental development. Pregnancy loss due to defects in implantation is a major cause of infertility in humans, and it is of clinical importance to identify the genes as well as the cellular and the molecular mechanisms that underlie this critical ECM/adhesion molecule-dependent crosstalk between conceptus and uterus (Norwitz et al. 2001). Recent global gene profiling using high-density microarray technology indicates that secreted phosphoprotein 1 (SPP1; also identified as osteopontin, bone sialoprotein 1 and early T-lymphocyte activation factor 1) is the most highly upregulated ECM-adhesion molecule in the human uterus as it becomes receptive to implantation (Carson et al. 2002, Kao et al. 2002, Mirkin et al. 2005).

SPP1 is a glycoprotein member of the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family of genetically related ECM proteins recognized as key players in diverse processes, such as bone mineralization,
cancer metastasis, cell-mediated immune responses, inflammation, and angiogenesis (Butler et al. 1996, Weber & Cantor 1996, Giachelli & Steitz 2000, Denhardt et al. 2003). SPP1 has potential to influence tissue remodeling at the conceptus–maternal interface by affecting cell–cell and cell–ECM communication, increasing cell proliferation, migration and survival, and regulating local cytokine networks (Johnson et al. 2003b). Indeed, temporal and spatial patterns of SPP1 expression within the uterus and the placenta of species with different placentation including rabbits, sheep, and humans suggest important roles during pregnancy. Depending on the species, SPP1 mRNA is induced in either the luminal epithelium (LE) or glandular epithelium (GE) during the peri-implantation period (Johnson et al. 1999a, 1999b, Apparao et al. 2001, 2003, von Wolff et al. 2001), and GE expression increases throughout pregnancy in the mouse pregnancy. Spp1 expression during the peri-implantation period has not been investigated completely (Nomura et al. 1992). Regardless of the placental type, the result is a consistent allocation of SPP1 protein to the conceptus/maternal interface where it is proposed to provide a conserved mechanism for stimulating changes in morphology and mediating adhesion between conceptus and uterine tissues essential for implantation and placentation (Johnson et al. 2003b).

There is evidence to suggest that Spp1 has a role(s) in mouse pregnancy. Spp1 null mice manifest decreased pregnancy rates during mid-gestation as compared with wild-type counterparts (Weintraub et al. 1988, Roux et al. 2000). Spp1 expression in the conceptus/maternal interface has been investigated in rabbits, sheep, and humans (Johnson 1999, Apparao et al. 2001, 2003, von Wolff et al. 2001). Spp1 expression during the peri-implantation period has not been investigated completely (Nomura et al. 1992). Therefore, as a basis for experiments to determine the mechanistic nature of critical functions of Spp1 during the peri-implantation period, the objectives of these studies were to describe the temporal and the spatial changes in Spp1 mRNA and protein in the mouse uterus during early gestation that may underlie the reproductive phenotype in Spp1 null mice, and to determine whether these changes can be attributed to alterations in steroid hormones.

Materials and Methods

Animals and tissue collection

All experimental and surgical procedures complied with the Guide for Care and Use of Laboratory Animals and were approved by the Texas A&M University Laboratory Animal Care Committee.

Temporal and spatial changes in Spp1 expression were evaluated in the uteri of adult-outbred CD-1 (Charles River Laboratories, Inc., Raleigh, NC, USA) mice during estrous cycle and pregnancy. Female mice were assigned randomly to cyclic or pregnant status, and those assigned to pregnant status were mated to intact fertile males of the same strain. Uteri were then collected during estrus, diestrus, and proestrus of the estrous cycle and on days 3, 4, 4.5, 5, and 6 of pregnancy (n=8 per day; day 1 defined as the day of vaginal plug).

Ovarian estrogen and progesterone advance the pre-receptive uterus to the receptive state, and the nidatory surge of estrogen initiates the attachment phase of implantation. Therefore, to examine the effects of progesterone and estrogen on Spp1 gene expression, female-outbred CD-1 mice were ovarioctomized and 2 weeks later injected subcutaneously with sesame oil (0.1 ml/mouse), estradiol (100 ng/mouse), progesterone (2 mg/mouse), or both estradiol and progesterone (n=8 per treatment) in sesame oil. Uteri were collected 24 h after treatment.

Several sections from uterine horns collected from one half of the mice for each treatment or day were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) and then embedded in Paraplast-Plus (Oxford Laboratory, St Louis, MO, USA). Uteri of the remaining mice from each treatment were embedded in Tissue-Tek optimal cutting temperature Compound (Miles, Oneonta, NY, USA), snap-frozen in liquid nitrogen, and stored at −80 °C prior to sectioning.

Real-time RT-PCR analysis

Total cellular RNA was isolated from frozen uteri using Trizol reagent (Invitrogen). Concentrations of Spp1 mRNA were determined by real-time RT-PCR. Primers were created from the murine Spp1 sequence (GeneBank Accession number X51834). Reactions contained 24 μl of the following reagents: 0.5 μl SPP1 forward primer from 10 μM solutions (sequence: TTTGTTGACTTTGTGTTGATCT), 0.2 μl Spp1 reverse primer from 10 μM solutions (sequence: GACAGGATGAGGGCAGTTGTAGTTCTTG(FAM); Invitrogen), 0.25 μl β-actin forward primer (Invitrogen), 0.25 μl β-actin reverse primer, 12.5 μl RT-PCR master mix (Invitrogen), 0.5 μl reverse transcriptase mix (Invitrogen), 0.5 μl ROX reference dye (Invitrogen), 1.5 μl MgCl₂, and 7.5 μl RNase-free water (Qiagen). Reactions also included 1 μl (100 ng) total RNA. The reverse primer of Spp1 was labeled with a reporter dye (FAM; D-LUX; Invitrogen) that allowed amplification of RNA to be evaluated on an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). The reaction conditions consisted of one cycle to reverse transcribe RNA (50 °C for 15 min then 95 °C for 2 min) followed by 45 cycles to amplify cDNA (95 °C for 15 s then 60 °C for 30 s). Appropriate controls were conducted to ensure that genomic DNA did not influence the amplification of the template. Following amplification, the RT-PCR product was analyzed on a 2% agarose gel to further validate size. The relative concentration of Spp1 mRNA was determined in the same samples via the comparative C₇ method with β-actin as the normalization control (JOE labeled.
certified LUX Primer Set; Invitrogen). Data were analyzed by assigning an arbitrary threshold cycle \( (C_T) \) for amplification plots, where the \( C_T \) for a sample was the cycle that its amplification plot crossed the threshold. The \( C_T \) was assigned in the log-linear range of amplification. The \( SPP1 \ \Delta C_T \) was determined by subtracting the \( \beta \)-actin \( C_T \) from the \( SPP1 \ \Delta C_T \) for each sample assayed. Calculation of the \( \Delta \Delta C_T \) involved using the highest \( \Delta C_T \) value as an arbitrary constant to subtract from all other \( \Delta C_T \) sample values. Fold-changes in gene expression are equivalent to \( 2^{-\Delta \Delta C_T} \).

**In situ hybridization analysis**

\( Spp1 \) mRNA was localized in paraffin-embedded mouse uterine tissue by *in situ* hybridization using methods described previously (Johnson et al. 1999a). Briefly, deparaffinized, rehydrated, and deproteinized uterine cross-sections (\( \sim 5 \) \( \mu \)m) were hybridized with radio-labeled antisense or sense mouse \( Spp1 \) cRNA probes (Fisher et al. 1995) synthesized by *in vitro* transcription with \([\alpha-\text{35S}]\)uridine 5-triphosphate (PerkinElmer Life Sciences, Wellesley, MA, USA). After hybridization, washes, and RNase A digestion, autoradiography was performed using nuclear track material type NTB-2 liquid photographic emulsion (Eastman Kodak, Rochester, NY, USA). Slides were exposed at 4°C for 5 days, developed in Kodak D-19 developer, counterstained with Harris’ modified hematoxylin (Fisher Scientific, Fairlawn, NJ, USA), dehydrated, and protected with cover slips.

**Immunofluorescence analysis**

For immunofluorescence staining of frozen sections, primary antibodies included rabbit anti-human recombinant \( SPP1 \) immunoglobulin (IgGs, LF-123 and LF-124; Fisher et al. 1995), a monoclonal mouse anti-CD45 IgG (eBioscience, San Diego, CA, USA), and a rat anti-macrophage IgG (F4/80; recognizes a 160 kDa membrane protein on mature macrophages, Hirsch & Gordon 1982). Secondary antibodies included fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Chemicon, Temecula, CA, USA), FITC-conjugated goat anti-rat IgG (Sigma), and Alexa 594-conjugated goat anti-rabbit and goat anti-mouse IgGs (Molecular Probes, Eugene, OR, USA).

Localization of \( Spp1 \) (without co-localization with other proteins) was performed as previously described (Johnson et al. 1999b). Briefly, frozen sections (\( \sim 10 \) \( \mu \)m) of uterus were cut with a cryostat (Hacker-BrightOTF, Hacker Instruments, Inc., Winnsboro, SC, USA) and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). Sections were fixed in methanol (\(-20^\circ\)C) for 10 min, air-dried, rehydrated at room temperature with 0.3% Tween 20 in 0.02 M PBS (rinse solution), and blocked in antibody dilution buffer (two parts 0.02 M PBS, 1.0% BSA, 0.3% Tween 20 (pH 8.0) and one part glycerol) containing 10% normal goat serum for 1 h at room temperature. Sections were then dipped in rinse solution at room temperature and incubated overnight at 4°C with 4 \( \mu \)g/ml \( SPP1 \) antibody (a cocktail containing LF-123 and -124 IgGs), and detected with FITC-conjugated secondary antibody. Slides were then overlaid with a coverglass and Prolong antifade mounting reagent (Molecular Probes).

For co-localization of proteins, frozen sections of uterus were cut, mounted, fixed, permeabilized, and blocked as described above. The sections were then dipped in rinse solution at room temperature and incubated overnight at 4°C with 2 \( \mu \)g/ml initial primary antibody (either anti-CD45 or anti-macrophage IgG). Following three washes in 4°C rinsing solution for 10 min each, sections were incubated with 2 \( \mu \)g/ml initial secondary antibody (either FITC-conjugated anti-rat or Alexa 594-conjugated anti-mouse IgG) for 4 h at room temperature, and washed in 4°C rinsing solution six times for 10 min each. The sections were then incubated overnight at 4°C with 4 \( \mu \)g/ml second primary antibody (a cocktail containing LF-123 and -124 IgG). Following six washes in 4°C rinsing solution for 10 min each, the sections were incubated with 2 \( \mu \)g/ml second secondary antibody (either FITC- or Alexa 594-conjugated anti-rabbit IgG) for 2 h at 4°C, washed six times in 4°C rinsing solution for 10 min each, and dipped in distilled–deionized H\( _2 \)O. Slides were overlaid with antifade mounting reagent as described above.

**Photomicrography**

Digital photomicrographs of representative bright- and dark-field images of *in situ* hybridization and representative fields of immunofluorescence staining were evaluated with a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY, USA) fitted with an Axioimaging HR digital camera. Digital images of *in situ* hybridization were recorded using AxioVision 3.0 or 4.3 software. For immunofluorescence co-localization of proteins, digital camera settings were evaluated to confirm that no ‘spectral bleed through’ FITC signal was detectable in the Alexa 594 filter set and vice versa. In these studies, once the distribution of individual antigens was established, the co-distribution of two antigens was investigated simultaneously in individual sections using compatible primary and FITC- or Alexa 594-secondary antibody combinations with appropriate filter sets. Individual fluorophore images were recorded sequentially with AxioVision 3.0 or 4.3 software and evaluated in multiple fluorophore overlay images recorded in the Zeiss Vision Image (ZVI) file format, which were subsequently converted to Tagged Image File (TIF) format. All *in situ* hybridization and immunofluorescence figures were assembled in Adobe Photoshop 7.0.1 (Adobe Systems Inc., San Jose, CA, USA).
USA). Any adjustment of brightness and/or contrast was applied uniformly to all images in a panel, and in the case of fluorescence images, to indicate the background fluorescence levels observed through the microscope.

**Statistical analysis**

Changes in concentrations of Spp1 mRNA were quantified by evaluating effects of reproductive status (estrus, diestrus, proestrus, days 5 and 6 of pregnancy) on the $\Delta C_T$ from real-time RT-PCR. Changes in Spp1 mRNA were analyzed in a randomized block design using a mixed model procedure (Proc Mixed procedure; SAS Inst. Inc., Cary, NC, USA), and reproductive status was included in the model as a fixed effect. No significant treatment effects were observed.

**Results**

**Expression of Spp1 mRNA in cyclic and pregnant mouse uterus**

In contrast to a previous report using northern blotting (Waterhouse et al. 1992), Spp1 mRNA was detected throughout the estrous cycle and through day 6 of pregnancy using the more sensitive methodology of RT-PCR (Table 1). However, no differences in Spp1 were evident between all days measured.

The presence of Spp1 within endometrial tissues necessitated localization of the Spp1 mRNA. In situ hybridization of mouse uterus localized Spp1 to LE and presumptive immune cells, but not to GE, stromal fibroblasts, vasculature, or myometrium. Although differences in total steady-state Spp1 mRNA were not evident by RT-PCR, changes in the distribution were clearly observed. During the estrous cycle, LE expression of Spp1 mRNA was prominent at proestrus, appeared to decrease by estrus, and was nearly undetectable at diestrus (Fig. 1). Spp1-expressing cells, later to be identified as immune cells (Fig. 4), were scattered within the stromal compartment of the endometrium throughout the estrous cycle. These cells were prominent at estrus (Fig. 1). During early pregnancy, Spp1 mRNA was present in endometrial immune cells similar to that observed during the estrous cycle (Fig. 2). However, Spp1 mRNA was not detected in LE until the evening of day 4, i.e., day 4.5 (Fig. 2). Expression of Spp1 mRNA in LE was transient, however, and was undetectable by day 6 of pregnancy, although immune cell expression remained prominent.

**Localization of Spp1 protein in pregnant mouse uterus**

Consistent with in situ hybridization results, immunoreactive Spp1 was prominent at the apical LE surface by day 5 of pregnancy (Fig. 3). In addition, Spp1 protein was localized in a pattern consistent with immune cells scattered within the endometrial stroma (Fig. 3).

**Initial characterization of Spp1-expressing endometrial immune cells**

Numerous cells scattered within the endometrium of pregnant mice showed positive immunostaining for Spp1 (Fig. 4A, top panel). In the same uterine cross-sections, similar cells immunoreacted with antiserum to the leukocyte-specific cell-surface marker CD45 (Fig. 4A, middle panel). When Spp1 (FITC conjugate) and CD45 (Alexa 594 conjugate) images were merged, individual cells within the same uterine cross-sections exhibited both Spp1 and CD45 immunoreactivity (Fig. 4A, bottom panel). All cells that stained strongly for Spp1 also expressed CD45; however, only a subset of CD45 cells expressed Spp1 (Fig. 4A).

Co-localization of immunostaining using anti-Spp1 LF-123/LF-124 and anti-macrophage F4/80 antisera showed that Spp1 was detectable in a subpopulation of endometrial macrophages (Fig. 4B). Whereas Spp1 was present in immune cells as well as at the apical surface of LE, macrophage immunoreactivity was limited to cells scattered within the endometrial stroma (Fig. 4B, top and middle panels). When Spp1 (Alexa 594 conjugate) and F4/80 (FITC conjugate) images were merged, the yellow fluorescence signal revealed the presence of Spp1 protein expression by some, but not all, macrophages (Fig. 4B, bottom panel).

<table>
<thead>
<tr>
<th>Reproductive status</th>
<th>$\beta$-actin $C_T^a$</th>
<th>SPP1 $C_T^a$</th>
<th>SPP1 $\Delta C_T^b$</th>
<th>SPP1 $\Delta \Delta C_T^c$</th>
<th>Fold difference$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrus</td>
<td>24.77±0.52</td>
<td>30.47±0.69</td>
<td>5.70±0.84</td>
<td>-1.69</td>
<td>3.2</td>
</tr>
<tr>
<td>Diestrus</td>
<td>24.89±0.52</td>
<td>30.30±0.79</td>
<td>5.41±0.98</td>
<td>-1.98</td>
<td>3.9</td>
</tr>
<tr>
<td>Proestrus</td>
<td>24.72±0.52</td>
<td>31.03±0.97</td>
<td>6.31±1.20</td>
<td>-1.08</td>
<td>2.1</td>
</tr>
<tr>
<td>Day 5</td>
<td>25.42±0.52</td>
<td>31.60±0.69</td>
<td>6.18±0.85</td>
<td>-1.21</td>
<td>2.3</td>
</tr>
<tr>
<td>Day 6</td>
<td>25.07±0.52</td>
<td>32.65±0.79</td>
<td>7.39±0.98</td>
<td>0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$^a$ $C_T$, the cycle that the amplification plot crossed an arbitrary threshold assigned in the log-linear range of amplification. Data presented as least square mean±s.e.m. $^b$ $\Delta C_T$, $C_T$ for target gene minus $C_T$ for normalization control, $\beta$-actin. Data presented as least square mean±s.e.m. $^c$ $\Delta \Delta C_T$, mean $\Delta C_T$ of a treatment minus the mean $\Delta C_T$ of the treatment with the least mRNA (which had the largest $C_T$). $^d$ Fold difference, $2^{-\Delta \Delta C_T}$, and are differences from the baseline expression of day 6.

Table 1: Quantitative RT-PCR analysis of SPP1 mRNA expression in the uterus.
Regulation of Spp1 in luminal epithelium by estrogen

Since both progesterone and estrogen play a role in regulating uterine receptivity for implantation, we examined whether these steroids differentially regulate the expression of Spp1 in the uterus. In ovariectomized mice, exogenous estrogen induced Spp1 mRNA expression in the uterine LE (Fig. 5). By contrast, progesterone was not effective in inducing uterine Spp1 expression (Fig. 5). Interestingly, co-treatment of ovariectomized mice with both estrogen and progesterone did not induce Spp1 expression in LE (Fig. 5). No changes were observed in the distribution of Spp1-positive immune cells with hormone treatment.

Discussion

Spp1 at the placental–endometrial interface

There is mounting evidence from studies in several species to support the hypothesis that SPP1, secreted by uterine epithelium, provides a substrate for integrin-mediated interactions at the conceptus–maternal interface (Johnson et al. 1999a, 1999b, Apparao et al. 2001, 2003, von Wolff et al. 2001). In each of these species, pregnancy-specific SPP1 expression in endometrial epithelium has been detected during the peri-implantation period. Although SPP1 protein is evident on the conceptus trophoderm and the apical surface of endometrial LE, SPP1 is not synthesized by the conceptus trophoderm in these species. The presence of SPP1 at this interface suggests an interaction with membrane-bound receptors on these respective tissues. It is noteworthy that previous reports have not implicated Spp1 as a mediator of implantation in...
mice (Waterhouse et al. 1992, Liaw et al. 1998). First, Spp1 initially escaped detection in mouse uterus between days 1 and 8 of pregnancy (Waterhouse et al. 1992). Secondly, no reproductive phenotype was detected during initial examination of mice with targeted disruption of the Spp1 gene (Liaw et al. 1998). Therefore, Spp1 has received only perfunctory interest with regard to the attachment of mouse blastocysts to uterine LE. However, recent publication of a highly provocative reproductive phenotype in Spp1-deficient mice identified peri-implantation defects (Weintraub et al. 2004). It was demonstrated that 72% of Spp1−/− mice with a verified copulatory plug were gravid on day 10.5 of gestation as compared with 38.5% of mice with some degree of disruption of the Spp1 gene. Further, litter sizes on 10.5, 15.5, and 19.5 days of gestation showed no effect of genotype on the number of embryos within mice that remained pregnant (Weintraub et al. 2004). A reasonable interpretation of these results is that disruption of the Spp1 gene leads to an incompletely penetrant, ‘all-or-nothing’ interference with either conception, implantation or the maintenance of gestation (Weintraub et al. 2004). Results reported here, for the first time, provide strong evidence that Spp1 is temporally and spatially localized within the pregnant mouse uterus to influence early events of implantation. These results show that Spp1 mRNA is transiently induced by estrogen in the endometrial LE of pregnant mice during the attachment phase of implantation, and that Spp1 protein is prominent at the apical LE surface. Therefore, Spp1 is now an excellent candidate gene for roles during the initial stages of mouse implantation.

In mice, ovarian progesterone and estrogen are crucial to render the uterus receptive to blastocyst implantation. These hormones act in a coordinated manner to temporally regulate proliferation and/or differentiation of specific cell types within the uterus. Preovulatory estrogen drives proliferation of epithelial cells. Rising concentrations of progesterone from the corpus luteum (CL) induce cell proliferation within the stromal compartment. Ovarian secretion of estrogen on the morning of day 4 of pregnancy, or nidatory estrogen, acts within this progestinized environment to further support
in mice, temporal changes in LE expression during the elongating pig conceptuses for pregnancy recognition, LE is a uterine response to estrogen secreted by investigations establish that estrogen regulates expression induction of Spp1 (White et al. 2001). The results of the present investigation suggest that estrogen treatment induced Spp1 mRNA expression in the endometrial luminal epithelium (LE). ST, endometrial stroma. Width of fields, 870 μm.

Figure 5 In situ hybridization analysis of Spp1 mRNA in cross-sections of mouse uteri collected from ovariectomized mice treated with sesame oil, estrogen, progesterone, or a combination of these steroids (estrogen + progesterone). The corresponding bright- and dark-field images of representative cross-sections of endometrium are shown. Note that estrogen treatment induced Spp1 mRNA expression in the endometrial luminal epithelium (LE). ST, endometrial stroma. Width of fields, 870 μm.

proliferation, while inducing LE to stop proliferating and begin differentiating into a functionally mature surface capable of responding to signals from the conceptus with the implantation reaction (Carson et al. 2000, Dey et al. 2004, Arman 2005). The results of the present investigation establish that estrogen regulates expression of Spp1 in mouse uterine LE. Although estrogen induction of Spp1 in LE during the peri-implantation period has previously been reported in pigs, the source of estrogen appears to be different between these species (White et al. 2005). Whereas porcine SPP1 expression in LE is a uterine response to estrogen secreted by elongating pig conceptuses for pregnancy recognition, in mice, temporal changes in LE expression during the estrous cycle and pregnancy are likely the result of ovarian estrogen. Spp1 mRNA is high in LE during proestrus when high concentrations of estrogen are unopposed by high concentrations of progesterone, in ovariectomized mice given exogenous estrogen, and in pregnant mice after the nidatory estrogen surge. As such, Spp1 can be grouped with lactoferrin and heparin-binding epidermal growth factor-like growth factor (HB-EGF) as prominent glycoproteins that increase in uterine LE in response to estrogen during the mouse peri-implantation period (Mc Master et al. 1992, Das et al. 1994, Wang et al. 1994).

The similarity of expression patterns between Spp1 and HB-EGF is particularly striking. For both the genes, there is a transient rise in expression within the uterine LE during the attachment phase of implantation. Expression is induced by administration of estrogen to ovariectomized mice, whereas no expression is observed with simultaneous estrogen and progesterone treatment (Wang et al. 1994). In contrast, although pregnancy is characterized by high concentrations of progesterone, the nidatory estrogen surge stimulates expression of both Spp1 and HB-EGF in LE (Das et al. 1994). Further, HB-EGF is not present in LE during progesterone-maintained delayed implantation, but is evident after activation of the blastocyst by estrogen injection (Das et al. 1994). Therefore, it is likely that the blastocyst influences LE expression of Spp1. The present data suggest that Spp1 expression in LE requires communication between an activated blastocyst and a uterus that is becoming implantation-receptive.

The potential for endometrial epithelial Spp1 to influence conceptus–maternal interactions is significant because the Arg-Gly-Asp (RGD) and other integrin-binding sequences of Spp1 interact with several high-affinity binding partners, including αvβ3, αvβ1, αvβ5, α4β1, α8β1, and α9β1 integrins (Hu et al. 1995a, 1995b, Denda et al. 1998, Smith & Giachelli 1998, Bayless & Davis 2001). A substantial body of work in the 1990s established that transient endometrial expression of αvβ3 and α4β1 integrins is cycle-dependent, and defines the window of implantation in women (Lessey et al. 1994), and that altered expression of these integrins correlates with several conditions associated with human infertility (Lessey et al. 1992, 1995). In livestock animals, multiple integrins have regulated and/or constituted expression at the apical surface of LE during the peri-implantation period (Bowen et al. 1996, Johnson et al. 2001).

Several RGD-containing integrins are also common features of pre- and peri-implantation blastocysts in mice. In mouse blastocysts, αvβ3 (often referred to as the Spp1 receptor) is present at the apical surface of trophectoderm cells, whereas α5β1 and α6β3 fibronectin receptors are localized intracellularly or at the basal surface of trophectoderm until integrin ligation promotes trafficking of α5β1 and α6β3 to strengthen fibronectin binding (Rout et al. 2004, Arman 2005). In mice, null mutations of αv, α5, β1, or β3 integrin genes

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result in peri-implantation lethality and failure of chorioallantoic membrane fusion, and functional blockade of αv and β3 integrins in mice and rabbits reduces the number of implantation sites (Hynes 1996, Illera et al. 2000, 2003). While a clear role for αvβ3 in mouse implantation has not been defined, it is possible that its ligation, perhaps to luminal Spp1, leads to apical translocation of α5β1 for stable conceptus attachment through fibronectin (Sutherland et al. 1993, Wang et al. 2000, Armant 2005). Indeed, αvβ3, α5β1, and αIIbβ3 appear in focal adhesions formed in trophoblast cells during outgrowth on fibronectin (Sutherland et al. 1993, Yelian et al. 1995, Wang et al. 2000). Further, trophoblast binding is attenuated by antibodies to the subunits αv and β3 (Schultz & Armant 1995, Illera et al. 2000, 2003, Rout et al. 2004). Finally, it appears that unoccupied αvβ3 transmits a positive cellular death signal and ligand interaction with this integrin protects cells from apoptosis (Zhao et al. 2005).

In addition to indirect effects on implantation, Spp1 may directly modulate conceptus cell proliferation, migration, survival, and attachment in mice (Standal et al. 2004). Both cell proliferation and apoptosis, which profoundly impact conceptus development during the peri-implantation period, are influenced by Spp1 (Khan et al. 2002, Celetti et al. 2005, Caers et al. 2006). Further, it is well documented that Spp1 binds integrins to influence cell attachment and migration (Senger & Perruzzi 1996, Smith & Giachelli 1998, Al–Shamiri et al. 2005, Celetti et al. 2005, Kolb et al. 2005, Caers et al. 2006). In addition, due to its flexible nature in solution and the prominence of polymerized Spp1 cross-linked by tissue transglutaminase, self-assembly of protein complexes to expose multiple RGD sequences for simultaneous interaction with integrins on trophoderm and LE to bridge conceptus to uterus is highly plausible (Fisher et al. 2001, Goldsmith et al. 2002). A model summarizing the present working hypothesis for the role of mouse uterine LE Spp1 during implantation is presented in Fig. 6.

**Spp1 in endometrial immune cells**

Despite previous speculation that localization of Spp1 mRNA and protein to cells scattered within the

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**Figure 6** Proposed model for roles of Spp1 in mediating trophoderm and endometrial interactions. The drawing is an adaptation of that used by Guillomot et al. (1993). Implantation in mice depends upon synchronized development of the embryo, so that it is competent to implant, and the uterus, so that it is receptive to embryo attachment, growth, and implantation. Ovarian estrogen (E2) and progesterone (P4) advance the pre-receptive uterus to the receptive state. Reciprocal signaling between the uterus and the embryo activate the embryo to become implantation competent. A result of the embryo–uterine communication is the induction of Spp1 in the uterine luminal epithelium (LE) after the nidatory surge of E2 at initial sites of embryo trophoderm attachment to the uterine LE for implantation. Spp1 is then secreted from LE where it can bind integrin receptors on trophoderm and/or LE to hypothesized: (1) bridge embryo to uterus during the transient phase of attachment for implantation, (2) initiate trafficking of α5β1 and αIIbβ3 integrins to strengthen embryo fibronectin binding, (3) inhibit embryonic apoptotic cell death, and (4) mediate embryo cell proliferation and migration essential for successful invasion of the uterine wall. Spp1 is also expressed by macrophages embedded within the stroma of both cyclic and pregnant mice. This Spp1 may contribute to the extensive endometrial remodeling that the uterus undergoes during the estrous cycle and early pregnancy.
endometrial stroma of sheep and pigs indicates production by immune cells (Johnson et al. 1999a, 2003b), results of the present study are the first to definitively localize immune cells that produce Spp1 within the peri-implantation endometrium of any species. The detection of a subpopulation of cells that express both CD45 and Spp1 agrees with a previous report of CD45-positive leukocyte isolation from human endometrium (von Wolff et al. 2001), and our results further suggest that, in mouse endometrium, at least some of these CD45-positive cells are a subset of macrophages. Certainly Spp1, also known as early T-lymphocyte activation factor-1, is an established component of the immune system. Reports of Spp1 activity in the immune system actually preceded its identification in malignant transformed cells and bone, Spp1 was later isolated independently from T-cells where it was the most abundantly increased gene in response to concanavalin-A treatment (Patarca et al. 1989). Spp1, associated with activated T-lymphocytes (Patarca et al. 1989, O’Regan et al. 1999), is strongly expressed by monocytes and macrophages (Takahashi et al. 2004), and is present in neutrophils (Ueno et al. 2001). It is not surprising that macrophages were identified in these studies. Activated macrophages are major cellular components of the uterus, the placenta, and the extraembryonic membranes that are capable of synthesizing and secreting a wide array of cytokines throughout pregnancy (Hunt 1990). Clearly, Spp1 is one of the products of activated macrophages within endometrial tissues. Although the expression of Spp1 by these multi-functional cells likely contributes to endometrial remodeling for implantation and placentation, at present, speculation for a specific role for Spp1 as a component of the endometrial immune system would be premature. However, it is generally accepted that macrophages are central to intricate hormone–prostaglandin–cytokine networks that regulate development within the pregnant uterus (Hunt 1990). In this context, Spp1 is a potentially powerful player. Immune cell Spp1 has roles in the maintenance and the remodeling of tissue during inflammation, where it mediates cell–cell and cell–ECM communication resulting in targeted chemotaxis of immune cells (Weber & Cantor 1996). In vitro, Spp1 can induce migration of both T-cells and macrophages (Patarca et al. 1989, Yamamoto et al. 1995), and in vivo infiltration of macrophages towards Spp1 injected into rat dermis has been observed (Giachelli et al. 1998). Importantly, Spp1 induces chemotactic migration of dendritic cells from the skin to the draining lymph nodes, a process required for many inflammatory responses (Weiss et al. 2001). Recently, Spp1 was shown to be a key component of Typ-1 (Th-1) immunity that upregulates interleukin (IL)-12 and downregulates IL-10 expression by macrophages and monocytes (Ashkar et al. 2000). Although beyond the scope of this initial investigation, future studies to carefully quantify both the percentage of each immune cell type that is positive for Spp1, as well as to document their temporal and spatial localization within the uterus during mouse pregnancy are warranted.

In conclusion, the hormonal regulation and exquisite temporal/spatial distribution of Spp1 at the apical surface of uterine LE during conceptus apposition provides a mechanism for Spp1 to bridge conceptus trophectoderm to uterine LE during the attachment period of implantation. Further, the presence of Spp1-positive macrophages within the underlying endometrial stroma suggests a role for Spp1 during remodeling of uterine tissue for conceptus invasion. The recent report showing that 49% of fewer Spp1 null mice maintain pregnancy through mid-gestation than wild-type counterparts yet show no difference in the numbers of implantation sites within mice that remain pregnant indicates that the loss of Spp1 at the maternal/fetal interface can be compensated for by other factors, but in half of the mice, disruption is severe enough to produce peri-implantation pregnancy failure. If pregnancy is maintained through this initial critical period, placentation proceeds in a grossly normal manner (Weintrub et al. 2004). The conserved expression pattern for SPP1 between humans, sheep, rabbits, and now mice adds considerable credibility to this idea (Johnson et al. 1999a, 1999b, Apparao et al. 2001, 2003, von Wolff et al. 2001). Results shown here significantly extend the physiological implications of Spp1 to mouse pregnancy, and should catalyze the future development of studies that focus on the mechanistic nature of functions of this component of the uterine/placental environment using the powerful genetic mouse model.

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