Expression of gastrin-releasing peptide (GRP) in the bovine uterus during the estrous cycle

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Summary. Gastrin-releasing peptide (GRP) has been proposed as a novel regulatory peptide in the reproductive tract. We previously demonstrated that GRP immunoreactivities are found predominantly in the uterine gland epithelial cells of nonpregnant and pregnant cows. The present study focused on the distribution of GRP immunoreactivity and the expression of GRP mRNA in the bovine endometrium during the estrous cycle. Tissues were collected from 21 uterine horns and bodies during the estrous cycle. RT-PCR showed the expected GRP mRNA fragments (284 bp) in the tissues from all stages of the cycle. *In situ* hybridization results ascertained the expression of the GRP mRNA in the uterine gland epithelial cells and superficial epithelial cells of the endometrium. Positive staining of GRP immunoreactivity in the uterine gland epithelial cells was detected in both the uterine horn and body from all stages of the cycle. In metestrus and diestrus stages, GRP was also detected in the superficial epithelial cells of horn, but not in the body. The degrees of GRP mRNA expression and intensities of GRP immunoreactivity in the endometrium increased from proestrus to diestrus stages. These findings suggest that GRP may be important both in the endometrial remodeling during the estrous cycle and in the implantation and development of blastocysts.

Introduction

The endometrium of the mature mammal undergoes both morphological and physiological changes in response to estrogen and progesterone (Hegele-Hartung *et al.*, 1992). These hormones influence endometrial cell function by either inducing the synthesis and secretion of other signaling molecules or modulating the presence of receptors for these agents. Substances produced and secreted by endometrial cells in response to estrogen or progesterone include prostaglandins (Jody *et al.*, 2000; Mann, 2001), regulatory peptides (Yallampalli *et al.*, 1998; Whitley *et al.*, 2000), cytokines (Red-Horse *et al.*, 2001), and opioids (Tseng *et al.*, 2000; Campos *et al.*, 2000). These substances then act in autocrine or paracrine manners to affect cellular events such as secretion, proliferation, and differentiation. Precise regulation of the endometrial function is necessary to maintain an internal environment of uterus suitable for reproductive events (Bartol *et al.*, 1999; Shemesh, 2001).

Gastrin-releasing peptide (GRP), originally found to be produced by intrinsic neurons of the gastrointestinal tract (McDonald *et al.*, 1979) as a homologue peptide of amphibian bombesin (McDonald *et al.*, 1978; Volkoff *et al.*, 1999), has been proposed as a novel regulatory peptide in the female reproductive tract (Happola *et al.*, 1989; Fraser *et al.*, 1994; Xiao *et al.*, 1996; Whitley *et al.*, 1996, 1998; Budipitojo *et al.*, 2001).

In the female reproductive tract, GRP exhibits various physiological effects such as the control of epithelial ion transport in the endometrium (Matthews *et al.*, 1993) and the modulation of uterine contraction (Amiot *et al.*, 1993) as well as an influence on the proliferation and differentiation of the endometrium (Endo *et al.*, 1991). It furthermore, serves as a circulating hormone in fetal and uterine devel-

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opment (Giraud *et al.*, 1993), as a mitogenic agent (Giraud *et al.*, 1996), and as an autocrine and paracrine regulatory peptide (Vetter and O'Grady, 1997) in the uterus.

In the ruminant uterus, this peptide is predominantly found in the epithelial cells of the endometrial gland (Fraser *et al.*, 1994; Whitley *et al.*, 1996, 1998; Budipitojo *et al.*, 2001), but also in the uterine fluid (Giraud *et al.*, 1994), suggesting secretion into the glandular lumen. However, in the nonpregnant uterus of ruminants, GRP immunoreactivity in the uterine gland cell has been reported in only one sample of a previous study on sheep (Fraser *et al.*, 1994). Furthermore, high levels of GRP mRNA are expressed only at day 16 in the estrous cycle of sheep (Whitley *et al.*, 1998), suggesting that the appearance of GRP in uterine gland epithelial cells of ruminants depends on the stages of the estrous cycle.

In our previous study of cows, various intensities of GRP immunoreactivity were localized in the uterine gland epithelial cells of all samples examined (Budipitojo *et al.*, 2001). However, a detailed examination of GRP distribution has yet to be carried out during the estrous cycle. The present study was thus designed to investigate the distribution of GRP immunoreactivity and GRP mRNA expression in the bovine uterus during the estrous cycle.

Materials and methods

Tissue samples

Twenty-one bovine uteri were used: four in proestrus, three in estrus, six in metestrus, and eight in diestrus stages of the

estrous cycle. The uteri were obtained from a local slaughterhouse as soon as possible after death. The tissues were collected from different areas of the caruncle and intercaruncle of the horn and body of the uterus. The tissues were then frozen in liquid nitrogen for reverse transcription followed by polymerase chain reaction (RT-PCR) analysis, and fixed in Bouin's fluid for histological and immunohistochemical evaluations. For *in situ* hybridization, uteri of the metestrus stage were cut into small blocks, embedded in OCT compounds (Miles Inc., Elkhart, USA), frozen in liquid nitrogen, and then stored at -80°C until use.

Immunohistochemistry

After Bouin's fixation, the specimens were dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Sections were cut serially at $5\text{ }\mu\text{m}$ in thickness and stained by hematoxylin and eosin for histological evaluation. The estrous cycle stages (proestrus, estrus, metestrus, and diestrus) were estimated based on the histological changes of the uterus in the cow as described by Priedkalns (1993).

For immunohistochemical staining, tissue sections were deparaffinized in xylene and rehydrated in a decreasing series of ethanol concentration. After washing in 0.01 M phosphate-buffered saline (PBS), endogenous peroxidase activity was blocked by incubating sections in 0.3% H_2O_2 in methanol for 15 min. The anti-GRP serum (R-6902, donated by Dr. N. Yanaihara, Yanaihara Institute, Shizuoka) was used as a primary antibody and was applied overnight at 4°C . The anti-GRP serum was raised in a rabbit by using synthetic porcine GRP as the antigen. A

Table 1. Distribution and semiquantitative evaluation of the intensity of the immunoreactivity of gastrin-releasing peptide in the bovine uterus during the estrous cycle

Portion of uterus	Proestrus	Estrus	Metestrus	Diestrus
Uterine horn:				
Superficial epithelium cells	—	—	±	+
Uterine gland epithelial cells				
Superficial glandular region	±	±	++	+++
Middle glandular region	±	+	+++	+++
Deep glandular region	+	++	++	±
Uterine body:				
Superficial epithelium cells	—	—	—	—
Uterine gland epithelial cells				
Superficial glandular region	±	±	+++	+++
Middle glandular region	±	+	+++	+++
Deep glandular region	+	++	++	±

+++ strong; ++ moderate; + weak; ± very weak; — not detected.

detailed characterization of the antiserum has been described elsewhere (Yanaihara *et al.*, 1981). The primary antibody was used at a final dilution of 1:9,000. To prevent nonspecific staining, sections were incubated with normal goat serum before incubation with the primary antibody. The secondary antibody, biotinylated anti-rabbit IgG raised in a goat (1:200, BA-1,000, Vector Laboratories Inc., Burlingame, CA, USA), was applied for 45 min at room temperature. The immunoreactive sites were visualized using the avidin-biotin peroxidase complex (ABC) method (Vectastain Elite® ABC Kit, PK-6100, Vector Laboratories) and a Tris-HCl buffer (pH 7.4) containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.03% H₂O₂. Stained sections were examined by conventional light microscope, and photomicrographs were taken with a digital camera (Coolpix 950, Nikon, Tokyo). The intensities of GRP immunoreactivities in uterine tissue were graded subjectively into 4 classes as shown in Table 1.

The specificity of the immunohistochemical staining was confirmed by two control conditions: 1) immunoabsorption of the primary antibody with a synthetic GRP (0.0001–100 µg/ml, R5553, Bachem, Torrance, CA, USA) prior to incubation of the section, and 2) replacement of the primary antibody with normal rabbit serum.

Isolation of RNA and analysis of messenger RNA (mRNA) by RT-PCR

Total RNA from the uterine horn was extracted from tissues by a single-step acid guanidium thiocyanate-phenol-chloroform extraction (AGPC) method (Chomczynski and Sacchit, 1987) by using ISOGEN (Wako, Osaka).

The first strand of cDNA was synthesized from 2 µl of the total RNA solution by using dT-3 Site Adaptor Primer and Avian Myeloblastosis Virus Reverse Transcriptase XL (Takara, Tokyo). For amplification of bovine GRP, the sense primer 5'-CTGGCCAAGATGTACACGCG-3' and anti-sense primer 5'-GTCCAAGAGTCGCACTTCCT-3' were used to generate a 284 bp of cDNA transcript (position 208–492 in the ovine GRP mRNA sequence as reported by Fraser *et al.* 1994). For PCR amplification, Takara Taq (Takara) was used according to the manufacture's instruction. The thermocycling condition of the PCR was denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. The amplified cDNA fragments were electrophoresed on 1% agarose gels in a 1×TAE buffer and then stained with ethidium bromide. The fragments then were observed using ultraviolet (UV) rays.

In situ hybridization

Two non-overlapping 45-mer antisense oligonucleotide probes were synthesized for bovine GRP cDNA. The oligonucleotides were labeled with ³⁵S-dATP to a specific activity of 0.5 × 10⁹ dpm/µg DNA, using terminal deoxynucleotidyl transferase (Promega, Madison, WI, USA). *In situ* hybridization analysis was performed as previously described (Mowa and Iwanaga, 2000). In brief, cryostat sections were fixed with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) for 15 min, and then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Hybridization was performed 42°C for 10 h by adding ³⁵S-labeled oligonucleotide probes at a final concentration of 0.35 × 10⁷ com/ml in 50% formamide containing 30 mM Tris-HCl (pH 7.6), 0.6 M NaCl, 1 mM EDTA, 100 mM dithiothreitol, 1×Denhardt's solution, 0.25% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 200 µg/ml yeast tRNA. Control hybridization was performed in the presence of a 20-fold excess amount of an unlabeled antisense probe. Slides were rinsed at room temperature for 30 min in 2×SSC (1×SSC is 150 mM NaCl, 15 mM sodium citrate) containing 0.1% sarkosyl, twice at 55°C for 40 min in 0.1×SSC containing 0.1% sarkosyl, dehydrated through a graded series of ethanol, and air dried. Slides were exposed to Hyperfilm β-max film (Amersham, Buckinghamshire, UK) for 2 weeks, or dipped into autoradiographic emulsion (NTB-2; Kodak, Rochester, NY, USA), exposed at 4°C for 4 weeks, and counterstained with hematoxylin. Sections were examined with a conventional light microscope, and photomicrographs were taken with a digital camera (Coolpix 950, Nikon, Tokyo).

Results

GRP expression

The results of RT-PCR showed that GRP mRNA was detectable in the endometrial tissues from all stages of the estrous cycle—proestrus, estrus, metestrus, and diestrus—as indicated by electrophoretic images of the RT-PCR fragments. The ethidium bromide staining and UV analysis showed that the RT-PCR produced fragments of GRP cDNA expected size 284 bp (Fig. 1). The intensities of electrophoretic images of the RT-PCR fragments increased from proestrus to diestrus.

For further confirmation of GRP mRNA expression, *in situ* hybridization was carried out in the metestrus stage of the uterus. *In situ* hybridization using two non-overlapping antisense oligonucleotide probes exhibited consistent labeling in the uterus. The specificity was confirmed by the

disappearance of the signals when an excess dose of the cold probe was added to the hybridization fluid. GRP mRNA was expressed predominantly in the uterine gland epithelial cells and in superficial epithelial cells, as clearly shown on X-ray films (Fig. 2a). Light microscopic observations on hybridized sections (microautoradiography) also revealed strong labeling in uterine gland epithelial cells and in the superficial epithelial cells (Fig. 2b, c). No significant signals were recognized in the uterus treated with the cold probe (Fig. 2d).

GRP distribution

Table 1 summarizes the distribution and semiquantitative evaluation of the intensity of immunoreactivities of GRP-positive cell in the bovine uterus during the estrous cycle. Positive staining of GRP was detected in all samples examined and predominantly observed in the supranuclear region of the uterine gland epithelial cells, both in the uterine horn and body. In contrast, no immunoreactivity for GRP was observed in the negative control.

The endometrium was separated into 3 regions to evaluate the localization of the GRP immunoreactivity in the uterine gland epithelial cells: the deep glandular region, middle glandular region, and superficial glandular region.

In the proestrus, no GRP immunoreactivity was detected in superficial epithelial cells of the horn (Fig. 3a) and body. In contrast, GRP immunoreactivity in epithelial cells of the uterine gland was very weakly detected in the superficial glandular region (Fig. 3a) and middle glandular region (Fig. 3b), and was weak in the deep glandular region of the horn (Fig. 3c) and body.

In the estrus stage, GRP immunoreactivity was also negative in the superficial epithelial cells of the horn (Fig. 4a) and body. In epithelial cells of uterine glands, GRP immunoreactivity was very weakly detected in the superficial (Fig. 4a) and middle (Fig. 4b) glandular regions, and was moderate in the deep glandular region (Fig. 4c) of the horn and body.

Very weak GRP immunoreactivity was localized in the superficial epithelial cells of the horn (Fig. 5a), but not in the body in the metestrus stage. GRP immunoreactivity in the epithelial cells of the uterine gland was moderately detected in the superficial (Fig. 5a) and deep glandular regions (Fig. 5c), and was strong in the middle glandular region (Fig. 5b) of the horn. Moreover, in the body, GRP immunoreactivities were strong in the superficial and middle glandular regions, and moderate in the deep glandular region.

In the diestrus, weak GRP immunoreactivity was detected in the superficial epithelial cells of the horn (Fig. 6a), but not in the body. GRP immunoreactivity in the epithelial

Fig. 1. Electrophoretic images of RT-PCR for GRP cDNA during estrous cycles. M: 1057-bp ladder marker. Lanes 1, 2, 3 and 4 show the results obtained from the bovine endometrium at the proestrus, estrus, metestrus and diestrus stages, respectively. Arrow indicates the specific product of 284 bp cDNA.

cells of the uterine gland was found strong in the superficial (Fig. 6a) and middle (Fig. 6b) glandular regions, but very weak in the deep glandular region (Fig. 6c) of the horn and body.

Discussion

The present study showed the expression of GRP mRNA in the bovine endometrial tissues from all stages of the estrous cycle using RT-PCR. Furthermore, the cellular site of GRP mRNA synthesis in the uterine gland epithelial cells and superficial epithelial cells of the metestrus stage was ascertained by an *in situ* hybridization method. Immunohistochemical studies also revealed the cellular localization of GRP peptide in the uterine gland epithelial cells during all stages of the estrous cycle and in the superficial epithelial cells during metestrus and diestrus stages.

As indicated by the electrophoretic images of RT-PCR fragments, it is likely that the degrees of GRP mRNA expression in endometrial tissues increase from proestrus to diestrus stages. These findings correlated with the increase in the intensity of GRP immunoreactivity in uterine gland epithelial cells and superficial epithelial cells of the endometrium from the proestrus to diestrus stages. The correlation between GRP mRNA and protein levels during the estrous cycle may indicate that, in the case of endometrial tissues of the cow, the degrees of GRP mRNA expression can be deduced simply from the intensity of GRP immunoreactivity.

The sites of GRP mRNA synthesis and GRP peptide storage in the uterine tissue of the cow during the estrous cycles are uterine gland epithelial cells and superficial epithelial cells of the endometrium. *In situ* hybridiza-

Fig. 2. *In situ* hybridization of GRP mRNA in the metestrus stage of the bovine uterus. **a:** X-ray images of the ^{35}S -labeled antisense probe show an intense expression of the mRNA in the uterine gland epithelial cells (thick arrow) and superficial epithelial cell (thin arrow) of the uterus. **b:** Light microscopic observation on a hybridized section (micro-autoradiography) reveals a strong expression of GRP mRNA in uterine gland epithelial cells (thick arrow) and superficial epithelial cells (thin arrow). **c:** Larger magnification of **b**. **d:** No significant signals are recognized in the uterus treated with the cold probe. a: $\times 40$, b: $\times 80$, c: $\times 320$

Fig. 3–6. Legends on the opposite page.

results showed the cellular sites of GRP mRNA synthesis in the uterine gland epithelial cells of all parts of the endometrium and in the superficial epithelial cells in the metestrus stage endometrium. This is consistent with the immunohistochemical results that revealed the GRP storage in the uterine gland epithelial cells from the deep, middle, and superficial glandular regions of the endometrium and in the superficial epithelial cells of the endometrium in the metestrus stage. The correlation of patterns of intensity between GRP immunoreactivity and GRP mRNA in the uterus has also been reported in sheep (Whitley *et al.*, 1998). These data indicate that immunohistochemical results generally reflect the changes seen with *in situ* hybridization. In the case of the bovine endometrium, it was suggested that GRP synthesis and storage were initiated in the glands farthest from the uterine lumen (deep glandular region) of the proestrus stage. The synthesis site extends up to the middle parts of the glands (middle glandular region) in the estrus stage. All parts of the uterine gland epithelial cells (deep, middle, and superficial glandular regions) and superficial epithelial cells actively produce and store GRP in the metestrus stage. At the diestrus stage, the end of GRP synthesis was initiated from the deep glandular region, extending to the middle and superficial glandular regions and superficial epithelial cells, respectively.

RT-PCR, *in situ* hybridization, and immunohistochemistry revealed that GRP mRNA expression and peptide localization in the ruminant uterus varied markedly during the estrous cycle, suggesting a regulatory phenomenon. The

increase in GRP immunoreactive intensity and GRP mRNA expression in the endometrium from proestrus to diestrus revealed in the present study of the cow accompanied the low GRP expression at days 4, 10, 12 and 14 of the estrous cycle, but then substantially increased at day 16 in the endometrium during the luteal phase of the normal cycles in the sheep (Whitley *et al.*, 1998), indicating that GRP induction is a cyclic phenomenon. Moreover, it has been known that GRP immunoreactivity is found in the uterine luminal fluids of pregnant sheep (Giraud *et al.*, 1994, 1996) and in the uterine gland secretions of both nonpregnant and pregnant cows (Budipitojo *et al.*, 2001). These data imply that GRP is secreted into the uterine lumen, and by its autocrine and paracrine regulatory capacity (Hajri *et al.*, 1992; Hill and McDonald, 1992; Vetter and O'Grady, 1997) participates in the regulation of cellular changes that occur in the uterus as a response to the increasing metabolic event that occurs during the estrous cycles. The cellular changes in the uterus caused by GRP could imply an increase in the electrogenic ion transport in the superficial epithelial cells of the endometrium (Matthews *et al.*, 1993; Vetter and O'Grady, 1997) and DNA synthesis in the stromal cells of the endometrium (Endo *et al.*, 1991). Furthermore, GRP also could serve as a mitogenic agent (Giraud *et al.*, 1996) and stimulate the secretion of other bioactive peptides (Carney *et al.*, 1987; Schubert *et al.*, 1992). These results suggest a possible role for GRP in endometrial remodeling and proliferation of the uterine epithelial cells during estrous cycles.

Fig. 3. Immunohistochemical staining for gastrin-releasing peptide (GRP) in the bovine endometrium in the proestrus stage. No GRP immunoreactivity is found in the superficial epithelial cells (thin arrow) of the uterine horn (a). In the uterine gland epithelial cells (thick arrow), very weak positive staining for GRP is detected in the superficial (a) and middle (b) glandular regions, and also a weak one in the deep glandular region (c) of the endometrium. a: $\times 320$; b, c: $\times 160$

Fig. 4. Immunohistochemical staining for gastrin-releasing peptide (GRP) in the bovine endometrium in the estrus stage. a: represents the lack of GRP immunoreactivity in superficial epithelial cells (thin arrow) of the horn endometrium. In epithelial cells of the uterine gland (thick arrow), immunoreactivity is very weakly detected in the superficial (a), weakly so in middle (b) and moderately so in the deep (c) glandular regions. a: $\times 320$; b, c: $\times 160$

Fig. 5. Immunohistochemical staining for gastrin-releasing peptide (GRP) in the bovine endometrium in the metestrus stage. Weak immunoreactivity is found in superficial epithelial cells (thin arrow) of the endometrium (a). Epithelial cells of the uterine gland (thick arrow) showing moderate immunoreactivity in the superficial (a) and deep (c) glandular regions, and strong immunoreactivity in the middle glandular region (b) of the uterine horn. a, b, c: $\times 160$

Fig. 6. Immunohistochemical staining for gastrin-releasing peptide (GRP) in the bovine endometrium in the diestrus stage. Weak immunoreactivity is found in the superficial epithelial cells (thin arrow) of the uterine horn (a). Epithelial cells of the uterine gland showing strong immunoreactivity (thick arrow) both in the superficial (a) and middle (b) glandular regions, and combination of very weak positive (thick arrow) and negative (thin arrow) immunoreactivity in the deep glandular region (c) of the uterine horn. a, b, c: $\times 160$

A comparison of the changes in GRP immunoreactivity and GRP mRNA expression between each of the estrous cycle stages suggests that the regulation of GRP gene expression may be related to ovarian steroid release. In the present study, the degree of GRP mRNA expression and the intensity of GRP immunoreactivity were optimal during the luteal phase when the progesterone level is high. This observation agrees with the study in sheep in that: 1) both GRP mRNA and peptide are elevated at day 16 of the cycle and day 20 of pregnancy (Whitely *et al.*, 1998); 2) there is a large induction of GRP expression that takes place during the luteolytic phase and the implantation stages of early pregnancy of sheep (Whitely *et al.*, 2000); and 3) constant and intense GRP-immunoreactive staining took place in uterine gland epithelial cells of the cow during pregnancy in our previous study (Budipitojo *et al.*, 2001), in the stage of a high level progesterone. Moreover, the expression pattern of GRP during the estrous cycle in the uterine gland epithelial cells revealed in this study coincided with the increase of the progesterone receptor level through proestrus and estrus, were maximal during metestrus, and decreased at the end of diestrus (Kimmins and MacLaren, 2001). These facts give the impression that progesterone acts as a positive regulator on the endometrial GRP expression. In contrast, the addition of progesterone to the ovariectomized ewe did not have any effect on endometrial GRP mRNA synthesis. Moreover, progesterone in combination with estrogen led to an enormous reduction in endometrial GRP mRNA compared with that in untreated ewes (Whitely *et al.*, 2000). This inconsistency between the endometrial GRP expression and the levels of estrogen and progesterone may be due to the combination of a temporal, tissue- and/or species-specific regulation of GRP expression by estrogen and progesterone.

In the bovine uterus, two types of cell were found to synthesize GRP mRNA: uterine gland epithelial cells of both the uterine horn and body, and superficial epithelial cells of the horn but not the body endometrium. The presence of GRP-immunoreactive staining and GRP mRNA expression in the superficial epithelial cells of the uterine horn but not in the body during metestrus suggests an important role for GRP in the implantation. It has been known that, in the ruminant uterus, the implantation process commonly happens in the uterine horn (Geary *et al.*, 1997; Bartol *et al.*, 1999). Our results showed that GRP was produced in the bovine endometrial tissues, and exactly known to be produced in the bovine uterine gland epithelial cells and superficial epithelial cells. The presence of high levels of GRP mRNA expression during metestrus, intense bands of the GRP mRNA, and strong GRP immunoreactivity during metestrus and diestrus in the uterine tissues of the present study was coupled with the known maximal secretion of

immunoreactive GRP into uterine luminal fluids just before attachment (Giraud *et al.*, 1994) and the presence of abundant GRP mRNA at day 16 of the ovine estrous cycle (Whitley *et al.*, 1998). These facts indicate that optimal GRP production in ruminant uterus during estrous cycle occurred in the luteal phase, when the uterine horn had already matured to accept blastocyst implantation. In the case of a successful implantation, the intensity of GRP immunoreactivity increased in sheep (Fraser *et al.*, 1994; Whitley *et al.*, 1998) and cows (Budipitojo *et al.*, 2001).

In conclusion, we confirmed the distribution of GRP immunoreactivity and expression of GRP mRNA in the bovine uterus during the estrous cycle. The present results suggest that GRP may be important both in endometrial remodeling during the estrous cycle and in the blastocyst implantation and development.

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