Proliferative Potential of Endometrial Stromal Cells, and Endometrial and Placental Expression of Cyclin in the Bovine

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Abstract. The objective of the present investigation was to study proliferative activity of fibroblast-like endometrial stromal cells in bovine endometrial caruncular (CAR) and intercaruncular (ICAR) areas that have distinct functions during implantation. Endometrial stromal cells were derived from CAR and ICAR of nonpregnant cows, and their proliferative potential was analyzed in an in vitro cell culture system. In addition, expression of four types of cell cycle regulatory molecules was analyzed by RT-PCR in samples of CAR, ICAR, cotyledon (COT) and fetal membrane of both artificially inseminated (AI) and somatic nuclear transferred (NT) cows on days 30 and 60 of gestation. The proliferation growth curve showed that the cells derived from CAR had higher proliferative activity than that of ICAR-derived cells. No morphological differences were found between the cells derived from CAR and ICAR at population-doubling levels (PDL) of the two. Most of the cells derived from ICAR of nonpregnant cows exhibited expanded shape with no further proliferation at PDL 6 with a lack of cyclin E expression. Of the regulatory molecules, cyclin D1, CDK2 and CDK4 were expressed in both CAR and ICAR cells derived from both nonpregnant, and AI cows on days 30 and 60 of gestation. The expression of cyclin E in both AI and NT cows was confined to COT and fetal membrane on day 30 of gestation. Cyclin E expression on day 60 of gestation in AI cows was observed in all but ICAR areas. In marked contrast, however, cyclin E expression on day 60 of gestation in NT cows was confined to COT, suggesting that poor placentation in these cows is possibly associated with a lack of cyclin E expression. These results suggest that CAR-derived stromal cells have higher proliferative potential, which may be related to cyclin E expression during implantation.

Key words: Bovine, Endometrium, Caruncle, Cell culture, Cyclin

poorly understood, the important processes such as implantation and placentation appear to be species specific. Decidual formation is an essential event required for implantation in humans and rodents [2], but in ruminants, caruncular (CAR) and intercaruncular (ICAR) areas of the endometrium exhibit discrete functions. CAR are nonglandular projections in the endometrium, mainly comprised of stromal cells and connective tissue [3], which is the site of implantation and placentation in ruminants. Intimate contact between maternal and fetal tissues occurs only in the CAR area, which later become placentome comprising fetal cotyledon and maternal caruncle [2]. Proliferative activity of stromal cells in CAR rapidly increases during implantation. Although the size of CAR in the nonpregnant uterus of the cow is approximately 10 mm or less in diameter, they attain 10 cm in diameter as placentomes are formed during pregnancy [3]. Factor(s) responsible for the induction of this rapid proliferation of CAR may be derived from the conceptus, since cell proliferation does not occur in nonpregnant uterus of cows, but little is known about the proliferative potential and mechanism of CAR in the bovine endometrium.

It is well known that cell proliferation progresses through cell cycle regulatory molecules, cyclins and cyclin-dependent kinases (CDKs) [4, 5]. Cyclins and CDKs form complexes by association with their corresponding partners, and the activated form of these molecules plays a critical role in the progression of cell cycle, resulting in cell proliferation [6, 7]. G1-cyclins such as cyclin D1 and E that are essential for G1/S progression play important roles in rhythmic proliferation of normal endometrium and development of the placenta in the human uterus [8, 9]. The cell cycle regulatory molecules are potentially involved in the rapid development of CAR after implantation in ruminants. Nevertheless, there are no data available to demonstrate the expression of these molecules and to compare the levels of expression of CAR and ICAR during the period of placentome formation. Investigation of the expression of cell cycle regulatory molecules such as cyclin family members may also provide insight into poor CAR development that has been associated with somatic nuclear transferred (NT) cows [10].

Therefore, the aim of the present study was to examine the potential of stromal cell proliferative activity in the bovine CAR and to compare it with that in ICAR in nonpregnant cows. Furthermore, together with stromal cell proliferation, cyclin expression was examined in the in vitro culture. Accordingly, the expression of cyclin D1 and E and their corresponding partners CDK4 and CDK2, respectively, were analyzed by RT-PCR on days 30 and 60 of gestation. The expression of these cell cycle regulatory molecules in the placenta of NT cows was also investigated.

**Materials and Methods**

**Preparation and cell cultures**

Bovine uteri during the functional luteal phase (approximately days 11–17) of the estrous cycle, based on ovarian morphology [11], were collected from a local slaughterhouse and transferred to the laboratory on ice. After washing in PBS (05913, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), the uterine horn was cut longitudinally and the uterine lumen was exposed. CAR and ICAR were easily distinguished by morphological examination of the luminal surface [12]. CAR and ICAR were physically dissected from the endometrium. Separation and purification of endometrial stromal cells were carried out according to the method described by Yamauchi et al. [13]. Briefly, Dulbecco’s Modified Eagle’s Medium and Ham’s F-12, 1:1 (v/v, DMEM/Ham’s F-12, D-2906, Sigma Chemical Co., St. Louis, MO, USA) containing 10% fetal bovine serum (FBS), 10 mM HEPES buffer solution (15630-080, Gibco BRL, Grand Island, NY, USA) and Penicillin-Streptomycin solution (P-0906, Sigma) were used as the principal culture medium. After washing, tissue pieces (1–2 mm³) were treated with 0.1% (w/v) collagenase (034-10533, Wako Pure Chemical Industries, Osaka, Japan) and incubated at 37 C for 1 h. Tissue fragments were then washed with the culture medium, resuspended and plated in 75 cm² tissue culture dishes (Falcon 3084, Becton, Deckinson and Company, Lincoln Park, NJ, USA). Primary culture cells were designated as population-doubling levels (PDL) of one. Epithelial cells were eliminated during the first and second passages. All cell culture procedures were done at 37 C in humidified air with 5% CO₂.

To examine the proliferation of endometrial stromal cells derived from CAR and ICAR, 1 × 10⁴ cells from each group were seeded in 48-well tissue
culture plates (Falcon 3078). After 2, 4, 6 and 8 days in culture, the total number of cells in each well was counted. Nuclei were extracted with High Resolution DNA Kit Type T (Partec, Münster, Germany) and labeled with fluorescent dyes; the number of cells was counted by calculating the total number of nuclei with a Ploidy Analyzer (Partec).

**Nuclear transfer and sample collections**

The endometrial tissues from somatic cell nuclear transferred (NT) cows were used for analysis of cell proliferative activity as a model to investigate aberrations in CAR development [10]. The NT embryos used in this study were derived from bovine ovarian cumulus cells according to the method described by Kato et al. [14]. Briefly, in vitro matured bovine oocytes were enucleated with a beveled glass pipette, and fused with donor cells by electric stimulation (single DC pulse; 25 V/150 µsec). The fused oocytes were treated with 2.5 µg/ml cytochalasin D (Sigma, St. Louis, MO, USA) plus 1µg/ml cycloheximide (Sigma) for 1 h, and 10 µg/ml cycloheximide alone for 4 more h. The NT embryos were cultured in CR1aa medium at 38.5 C in an environment of 5% CO2, 5% O2, and 90% N2. On day 7, good quality blastocysts were transferred nonsurgically into synchronized recipient Japanese black cows [15]. Normal pregnancies were attained by artificial insemination (AI) of Japanese black cows. NT and AI cows were slaughtered around day 30 (days 27–34) and day 60 (days 60–64) of gestation (n=3 per group). Fetal and maternal portions of the placentome such as cotyledon and caruncle, and intercotyledonary fetal membrane and intercaruncular endometrium were collected separately for RNA extraction. The cotyledon was mechanically separated from the uterine caruncle. All samples were snap frozen in liquid nitrogen and stored at –80 C until RNA extraction.

**Reverse transcription (RT) and amplification of cDNA**

Total RNA from bovine placental and uterine tissues was isolated with ISOGEN (Nippon Gene CC, Toyama, Japan) according to the manufacturer’s instructions. After determination of RNA purity, and concentration with a spectrophotometer, two µg of total RNA was used for reverse transcription in a 20 µl reaction volume. The total RNA was reverse transcribed with 200 units of Super ScriptTM II reverse transcriptase (Gibco BRL, Life Technologies, Inc., New York, USA) in the presence of 0.5 µg of oligo dT primer, 500 µM dNTP, 2.5 mM MgCl2 and 10 mM DTT at 42 C for 50 minutes. After heat inactivation of reverse transcriptase at 70 C for 5 minutes, 2 units of ribonuclease H was added to the reaction mixture and incubated at 37 C for 20 minutes before polymerase chain reaction (PCR). The PCR primer sequences and the predicted cDNA sizes of amplified products are listed in Table 1. The primers for CDK4, cyclin D1 and cyclin E were designed from the mouse sequences: GenBank accession No; NM_009870, NM_007631 and NM_007633, respectively. The primers for CDK2 were designed from the human sequence (GenBank accession No: X62071). PCR was performed with a thermal cycler (MJ Research, inc., Watertown, MA, USA) and the reaction mixture consisted of cDNA template, 0.5 µM primers, 0.2 mM dNTP mixture and 0.1 units/µl AmpliTaq gold DNA polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA). PCR conditions were as follows (in order of denature, annealing and extension): 95 C for 30 sec, 55 C for 30 sec and 72 C for 60 sec. Then 10 µl of PCR product and 1 µl of 10 × loading buffer were mixed and electrophoresed at 100 V on a 2% agarose gel in TAE buffer (40 mM Tris-acetate, 10 mM EDTA, pH 7.8), and PCR products were visualized by ethidium bromide staining.

**Statistical analysis**

Proliferation of cells derived from CAR or ICAR was analyzed by Student’s unpaired t-test. Differences were considered significant at P<0.05.

**Results**

When bovine endometrial stromal cells were

<table>
<thead>
<tr>
<th>CDKs and cyclins</th>
<th>Primer sequences</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK2</td>
<td>5’CATCCCTCTCCCCCTCCTACAA3’</td>
<td>491</td>
</tr>
<tr>
<td></td>
<td>5’AGCTCCGTCATCCCATCTACCC3’</td>
<td></td>
</tr>
<tr>
<td>CDK4</td>
<td>5’CCTTCCCGTACGACATTCGC3’</td>
<td>393</td>
</tr>
<tr>
<td></td>
<td>5’CAGAGCTTAACCCCTAACGAG3’</td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>5’TGGAGGGGCGTGGAAGAGCG3’</td>
<td>332</td>
</tr>
<tr>
<td></td>
<td>5’GGAGGCTGGTCTGGGAAATGA3’</td>
<td></td>
</tr>
<tr>
<td>Cyclin E</td>
<td>5’TTCCTTGGATTGCGGTAGGAG3’</td>
<td>462</td>
</tr>
<tr>
<td></td>
<td>5’AAGCAGCGAGGGACACCATAA3’</td>
<td></td>
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seeded onto 48-well culture plates at a concentration of $1 \times 10^4$ cells/well, they proliferated to a confluent state in 5–6 days. Figure 1 shows proliferation growth curves of the cells derived from CAR and ICAR. The number of CAR derived cells in each well was significantly ($P<0.05$) higher than that of ICAR derived cells two days after seeding cells. After 4, 6 and 8 days in culture, the number of CAR derived cells was 2 to 3 times greater than that of ICAR derived cells.

Bovine endometrial stromal cells were a monolayer of spindle- and fibroblast-like cells in primary culture. No morphological differences were observed between cells derived from CAR and ICAR at PDL 2 (Fig. 2a A and D), and the cells were crowded and took on a narrow spindle shape. Although the morphology was maintained in cells derived from CAR (Fig. 2a B) until PDL 4, some cells from ICAR showed signs of senescence with expanded shape and a large nucleus (Fig. 2a E). Most of the ICAR-derived cells became expanded with no further proliferation at PDL 6 (Fig. 2a F). Expression of cyclin D1 and cyclin E mRNA in cultured bovine endometrial stromal cells analyzed by RT-PCR is shown in Figure 2b. Cyclin D1 mRNA was detected in both CAR- and ICAR-derived cells in all PDL that we examined, but the bands of cyclin E mRNA became weak as the PDL increased. In ICAR-derived cells at PDL 6, cyclin E mRNA was not detectable and the cells exhibited expanded shape with no further proliferation (Fig. 2a F).

Expression of CDK and cyclin mRNAs in the bovine placenta and uterus on days 30 and 60 of gestation in AI cows was analyzed by RT-PCR. All PCR products amplified with specific primers were found to be of the sizes predicted from mouse and human sequences; CDK2: 491 bp; CDK4: 393 bp; cyclin D1: 332 bp; cyclin E: 462 bp. CDK2, CDK4 and cyclin D1 mRNA were detected in all four different tissue types examined. But cyclin E mRNA was detected only in cotyledonary tissues and intercotyledonary fetal membrane on day 30 whereas its expression was observed also in the caruncular tissues on day 60 of gestation, but not in the intercaruncular endometrium (Fig. 3, Table 2). Expression of cyclin mRNAs in NT cows on days 30 and 60 of gestation was examined and compared with that of AI cows. Cyclin D1 mRNA was detected in the placenta and uterus in both NT and AI cows regardless of gestation age. On the other hand, cyclin E expression on day 30 of gestation was similar in AI and NT cows in which the expression was detected only in fetal membrane and some small cotyledon. In a marked contrast, cyclin E expression on day 60 of gestation in NT cows was detected only in cotyledon.

**Discussion**

To the best of our knowledge, this is the first report to show the differences in proliferative potential of stromal cells derived from CAR and ICAR in nonpregnant cows and in *in vitro* cultured cells, and also to demonstrate cyclin E expression in placental tissues derived from both artificially inseminated (AI) and somatic nuclear recipient (NT) cows. In this study, the proliferative potential of CAR was analyzed and compared with that of ICAR at both the molecular and cellular levels. The...
Proliferation of bovine caruncular cells

proliferative activity of CAR-derived stromal cells was found to be high and the activity appears to be associated with the expression of a cell cycle regulatory molecule, cyclin E. This was based on the observations that cyclin E continued to express in nonpregnant CAR-derived stromal cells until PDL 6, and its expression was also evident in CAR area of placenta in AI cows. Interestingly, as opposed to AI cows, cyclin E expression was not observed in maternal tissues of placenta in NT cows as gestation advanced, suggesting that poor CAR development frequently observed in NT cows is likely associated with a lack of cyclin E expression.

There are numerous reports that investigated the functions of the bovine uterus by using in vitro cultured cells derived from endometrium. Xiao and Goff [16] analyzed the effect of steroid hormones on the number of progesterone and estradiol receptors in primary cell cultures of bovine endometrial stromal and epithelial cells. Davidson et al. [17] investigated the effect of interferon-tau, a unique interferon secreted by ruminant conceptus, on proliferation of bovine endometrial stromal and epithelial cells. Although these studies have contributed to our understanding of the functions of endometrial cells in general, the source of these stromal cells had not been distinguished despite

Fig. 2. Culture of endometrial stromal cells derived from caruncle (CAR) and intercaruncular endometrium (ICAR). (a) Phase-contrast observation. CAR derived cells (A–C) and ICAR derived cells (D–F). Population-doubling levels (PDL) of 2 (A, D), PDL 4 (B, E) and PDL 6 (C, F). Scale bars represent 500 µm. (b) Expression of cyclin D1 and cyclin E mRNA in cultured bovine endometrial stromal cells, as analyzed by RT-PCR. Lane 1 and 4: PDL 2; lane 2 and 5: PDL 4; and lane 3 and 6: PDL 6.
The endometrium is one of the remarkable remodeling tissues, and the renewal of cells depends on the balance between cell proliferation and cell death including apoptosis during this remodeling process. During implantation, placentomes develop specifically from CAR in the cow, though no specific regulatory mechanism has been documented. The present study demonstrated that the stromal cells derived from CAR have higher proliferative activity than that of ICAR-derived stromal cells and that this activity appeared to be related to the expression of the cell cycle regulatory molecule, cyclin E. This is also supported by the present observation that very low levels of expression of cyclin E are found in ICAR of pregnant cows on days 30 and 60 of gestation. These data do not provide direct evidence that cyclin E is the primary cause that drives development of CAR. It should be emphasized, however, that cyclin E is a potential candidate in this respect because cyclin E can accelerate the transition from G1 to the S phase during normal cell proliferation. In this study, endometrial stromal cells from cows were collected during the luteal phase of the estrous cycle because bovine endometrium during this period of the estrous cycle is receptive to embryos and allows pregnancy to be sustained after embryo transfer [19]. Furthermore, the endometrium during this period of the estrous cycle is in the stage at which levels of various factors such as steroid hormones and cytokines are similar to those in early gestation [1, 20, 21].

The proliferating activity of stromal cell may depend on steroid hormones acting through cyclin E [22, 23]. Some factors such as TGF-beta may regulate cell proliferation through P27, the cell-cycle inhibitor, but no evidence has been

Table 2. RT-PCR analysis of cyclin D1 and E mRNA expression in placenta and uterus of NT and AI cows

<table>
<thead>
<tr>
<th>Cyclin</th>
<th>Day of gestation</th>
<th>NT cows</th>
<th>AI cows</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>COT</td>
<td>ICOT</td>
<td>CAR</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>30*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>30*</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Positive bands were detected; – Bands were not detected. *At day 30 of gestation, COT contained both COT and ICOT tissues because of difficulty in mechanical separation. NT, somatic cell nuclear recipient; AI, artificial insemination; COT, cotyledon; ICOT, intercotyledonary fetal membrane; CAR, caruncle; ICAR, intercaruncular endometrium.
documented on the bovine endometrium [9, 23]. These regulatory mechanisms may be related to hormonal signals, in particular, higher levels of progesterone during gestation [22, 23]. In the present study, the expression of cyclin E in endometrium of NT cows was minimal and this evidence coincided with our previous finding that caruncular development in NT cows was very poor [10]. Nevertheless, this finding has a strong implication in cattle in contrast to other species such as humans or rodents, because the attachment and placentome formation between the embryonic tissues and endometrium occur only in the caruncular area in cattle. Cyclin E expression was detected in CAR and ICAR areas of the endometrium during the estrous cycle (data not shown); but the expression was found only in CAR but not in ICAR on day 60 of gestation. It may be that cell proliferation inhibitor such as TGF-beta have a suppressive effects on ICAR proliferation, as has been shown in human endometrium [22], and perhaps some other inhibitory molecules expressed in ICAR during gestation might also be involved [24]. Nevertheless, there is no direct evidence for TGF-beta, which may originate in either ICAR itself or fetal tissues, to play such a role in the inhibition of ICAR proliferation. Further studies are necessary to confirm this hypothesis as far as bovine endometrium is concerned.

It is reported that the cell cycle regulatory molecules play important roles in human endometrium during the menstrual cycle and placental development [25, 26]. Shiozawa et al. [8, 22] reported that cyclin E, CDK2 and CDK4 are highly expressed in the glandular and stromal cells during the proliferative phase of the menstrual cycle. Bamberger et al. [9] suggested that complex expression patterns of cyclin E, and p27, an inhibitor of CDKs, control the mechanism of human placental proliferation and TGF-beta may participate in the inhibitory process during cell proliferation. In this study, in addition to cotyledonary and intercotyledonary fetal membranes, expression of cyclin E was found in CAR but not in ICAR on day 60 of gestation. This finding suggests that cyclin E is involved in the promotion of CAR/placenta development after implantation. Since cyclin E expression was not observed, it is possible that cell cycle inhibitors are expressed in the ICAR. Further studies are required to define how cell cycle regulatory molecules control the mechanism of CAR development after implantation.

Expression of cyclin E in CAR appears to be related to the activity or growth of the conceptus, because in the present study, cyclin E was not expressed in CAR of NT cows on day 60 of gestation. An abnormal and/or poorly developed placenta can generally be observed in NT cows. A previous report [10] from this laboratory shows that NT cows not only have fewer placentomes but also have poorly developed caruncles. The present data also suggest that unsuccessful placentation in NT cows is most likely associated with a lack of cyclin E expression in CAR. Similarly, the lack of cyclin E expression in intercotyledonary fetal membrane of NT cows probably reflects a dormant condition of cell activity resulting from lower cell proliferation activity. The absence of cyclin E expression in intercotyledonary fetal membrane of NT embryos on days 30 and 60 of gestation supports the delay of NT embryo development reported in previous reports [10, 27]. Further studies are required to determine the factor(s) that induce cyclin E expression in fetal as well as maternal with special reference to CAR tissue during placentation.

In conclusion, we demonstrated a few distinct proliferative properties of stromal cells in CAR which are different from the stromal cell-characteristics of ICAR, at both the molecular and cellular levels. It is suggested that cyclin E is likely to be a major candidate that promotes placental development after implantation, and that poor placentation in somatic cell nuclear recipient cows is possibly associated with a lack of cyclin E expression in the maternal tissues. Therefore, the present data provide useful information to define some regulatory mechanisms of implantation and placentation.

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References


